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(54) Title: FY7 POLYMERASE

(57) Abstract

A purified recombinant thermostable DNA polymerase which exhibits at least about 80 % activity at salt concentrations of 50 mM and greater, at least about 70 % activity at salt concentrations of 25 mM and greater, and having a processivity of about 30 nucleotides per binding event. An isolated nucleic acid that encodes the thermostable DNA polymerase, as well as a recombinant DNA vector comprising the nucleic acid and a recombinant host cell transformed with the vector, are also disclosed. A method of sequencing DNA using the DNA polymerase as well as a kit for sequencing DNA is also disclosed.

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FY7 POLYMERASE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to United States Provisional Application Serial No. 60/089,556, filed on June 17, 1998, the entire disclosure of which is incorporated in its herein.

BACKGROUND OF THE INVENTION

Field of the Invention

The instant disclosure pertains to thermostable DNA polymerases which exhibit improved robustness and efficiency.

Background

DNA polymerases are enzymes which are useful in many recombinant DNA techniques such as nucleic acid amplification by the polymerase chain reaction ("PCR"), self-sustained sequence replication ("3SR"), and high temperature DNA sequencing. Thermostable polymerases are particularly useful. Because heat does not destroy the polymerase activity, there is no need to add additional polymerase after every denaturation step.

However, many thermostable polymerases have been found to display a 5' to 3' exonuclease or structure-dependent single-stranded endonuclease ("SDSSE") activity which may limit the amount of product produced or contribute to the plateau phenomenon in the normally exponential accumulation of product. Such 5' to 3' nuclease activity may contribute to an impaired ability to efficiently generate long PCR products greater than or equal to 10kb, particularly for G+C rich targets. In DNA sequencing applications and cycle sequencing applications, the presence of 5' to 3' nuclease activity may contribute to a reduction in desired band intensities and/or generation of spurious or background bands.

Additionally, many of the enzymes presently available are sensitive to high salt environments, a condition commonly

Presently available enzymes have so-so processing ability (are more distributive - fall off more often – explain in more detail)

dITP added to address compression problems – usually kills activity of enzyme

Thus, a need continues to exist for an improved DNA polymerase having increased tolerance to high salt conditions, efficient utilization of dITP, high productivity, and improved performance on GC-rich templates.

BRIEF SUMMARY OF THE INVENTION

The instant disclosure teaches a purified recombinant thermostable DNA polymerase comprising the amino acid sequence set forth in Figure 1, as well as a purified recombinant thermostable DNA polymerase which exhibits at least about 80% activity at salt concentations of 50 mM and greater. The instant disclosure further teaches a purified recombinant thermostable DNA polymerase which exhibits at least about 70% activity at salt concentrations of 25 mM and greater, and a purified recombinant thermostable DNA polymerase having a processivity of about 30 nucleotides per binding event.

The instant disclosure also teaches an isolated nucleic acid that encodes a thermostable DNA polymerase, wherein said nucleic acid consists of the nucleotide sequence set forth in Figure 1, as well as a recombinant DNA vector that comprises the nucleic acid, and a recombinant host cell transformed with the vector.

The instant disclosure also teaches a method of sequencing DNA comprising the step of generating chain terminated fragments from the DNA template to be sequenced with the DNA polymerase in the presence of at least one chain terminating agent and one or more nucleotide triphosphates, and determining the sequence of said DNA from the sizes of said fragments. The instant disclosure also teaches a kit for sequencing DNA comprising the DNA polymerase.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIGURE 1 depicts the amino acid sequence (and DNA sequence encoding therefor) for the FY7 polymerase.

FIGURE 2 depicts the DNA sequence of M13mp18 DNA sequenced using the FY7 polymerase formulated in Mn conditions, as shown by a print out from an ABI model 377 automated fluorescent DNA sequencing apparatus.

FIGURE 3 depicts the DNA sequence of M13mp18 DNA sequenced using the FY7 polymerase formulated in Mg conditions, as shown by a print out from an ABI model 377 automated fluorescent DNA sequencing apparatus.

FIGURE 4 depicts the percent of maximum polymerase activity for Thermo Sequenase™ enzyme DNA polymerase versus FY7 DNA polymerase under varying KCl concentrations.

FIGURE 5 depicts the effect of high salt concentrations on DNA sequencing ability in radioactively labeled DNA sequencing reactions using Thermo Sequenase™ enzyme DNA polymerase versus FY7 DNA polymerase.

FIGURES 6-10 depict the effect of increasing salt concentration on the performance of Thermo Sequenase. At concentrations as low as 25mM data quality is affected with the read length being decreased from at least 600 bases to about 450 bases. At 50mM salt the read length is further decreased to about 350 bases, 75mM to about 250 bases and at 100mM the read length is negligible.

FIGURES 11-15 depict the effect of increasing salt concentration on the performance of FY7 DNA polymerase. There is no detrimental effect on performance to at least 75mM KCl and only a slight decrease in data quality at 100mM KCl.

FIGURE 16 depicts the processivity measured for Thermo Sequenase DNA polymerase, AmpliTaq FS DNA polymerase, compared with the processivity measured for FY7 DNA polymerase.

FIGURE 17 depicts the improved read length obtained when using FY7 polymerase versus Thermo Sequenase DNA polymerase in radioactively labeled sequencing reactions incorporating the dGTP (Guanosine triphosphate) analog dITP (Inosine triphosphate) at 72 °C.

FIGURES 18-22 show the effect of increasing extension step time on the read length and data quality produced by Thermo Sequenase DNA polymerase in fluorescently labeled terminator DNA sequencing reactions

FIGURES 23-27 show the effect of increasing extension step time on the read length and data quality produced by FY7 DNA polymerase in fluorescently labeled terminator DNA sequencing reactions.

DETAILED DESCRIPTION OF THE INVENTION

A series of polymerase mutants were constructed with the aim of obtaining an improved polymerase for DNA sequencing, by reducing the exonuclease activity found in full length *Thermus thermophilus* and *Thermus aquaticus* DNA polymerase I enzymes. Six conserved motifs (Gutman and Minton (1993) Nucleic Acids Research 21, 4406 - 4407) can be identified in the amino-terminal domain of pol I type polymerases, in which the 5' to 3' exonuclease activity has been shown to reside. Further, six carboxylate residues in these conserved regions have been shown in a crystal structure to be located at the active site of the exonuclease domain of *Thermus aquaticus* DNA pol I (Kim et al., (1995) Nature 376, 612-616). Point mutations were made by site-directed mutagenesis to carboxylates and other residues in three of six conserved motifs in Tth and Taq polymerases as follows:

Taq D18A, Taq T140V, Taq D142N/D144N. All of these have the mutation F667Y outside of the exonuclease domain.

Tth D18A, Tth T141V, Tth D143N/D145N. All of these have the mutation F669Y outside of the exonuclease domain.

All polymerases were evaluated for exonuclease activity, processivity, strand displacement, salt tolerance, thermostability, and sequencing quality. One FY7 polymerase, Tth D18A, F669Y, is described in further detail below.

EXAMPLES

Methods

In vitro mutagenesis

PCR was employed to introduce an aspartic acid to alanine amino acid change at codon 18 (D18A) of cloned full length F669Y Tth (plasmid pMR10). Mutagenic Primer 1 (CTGTTCGAACCCAAAGGCCGTGTCCTCCTGGTGGCCGGCCACCAC) spans nucleotides 19-60 of pMR10 including codon 18 and a *Bst*BI restriction site. Oligonucleotide Primer 2 (GAGGCTGCCGAATTCCAGCCTCTC) spans an *Eco*RI site of pMR10. pMR10 was used as template DNA. The PCR product was digested with *Bst*BI and *Eco*RI and ligated to two fragments of pMR10: a 5000 bp *KpnI/ Bst*BI and a 2057 bp *Eco*RI / *Kpn*I, creating plasmid pMR12. Cells of *E. coli* strain DH1λ^+ were used for primary transformation, and strain M5248 (λ cI857) was used for protein expression, although any comparable pair of *E. coli* strains carrying the cI^+ and cI857 alleles could be utilized. Alternatively, any rec^+ cI^+ strain could be induced by chemical agents such as nalidixic acid to produce the polymerase.

Purification of Polymerase

M5248 containing plasmid pMR12 was grown in one liter of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl), preferably 2X LB medium, containing 100 mg/ml ampicillin at 30°C. When the OD_{600} reached 1.0, the culture was induced at 42°C for 1.5 hours. The cultures were then cooled to <20°C and the cells harvested by centrifugation in a Sorvall RC-3B centrifuge at 5000 rpm at 4°C for 15 to 30 minutes. Harvested cells were stored at -80°C.

The cell pellet was resuspended in 25 ml pre-warmed lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 1 mM EDTA, 0.1%, preferably 0.2% Tween 20, 0.1%, preferably 0.2% NP40). Preferably, the lysis buffer contains 300 mM NaCl. Resuspended cells were incubated at 75 - 85°C for 10-20 minutes, sonicated for 1 minute, and

cleared by centrifugation. The cleared lysate was passed through a 300 ml column of diethylaminoethyl cellulose (Whatman DE 52) equilibrated in buffer A (50mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% Tween 20, 0.1% NP40) containing 100mM, preferably 300 mM NaCl. Fractions were assayed for polymerase activity, and those demonstrating peak polymerase activity were pooled, diluted to 50 mM NaCl with Buffer A, and loaded onto a heparin sepharose column (20 ml) equilibrated with 50 mM NaCl in buffer A. The polymerase was eluted from the column with a linear salt gradient from 50 mM to 700mM NaCl in buffer A. Fractions were assayed for polymerase activity, and those demonstrating peak activity were pooled and dialyzed against final buffer (20mM Tris-HCl pH8.5, 50 % (v/v) glycerol, 0.1mM EDTA, 0.5% Tween 20, 0.5% NP40, 1mM DTT, 100mM KCl). The purified protein is designated FY7. The amino acid sequence (and DNA sequence encoding therefor) are presented in Figure 1.

Bacterial Strains

E. coli strains: DHIλ^+ [gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44, λ^+]; M5248 [λ (bio275, cI857, cIII+, N+, λ (H1))].

PCR

Plasmid DNA from *E. coli* DHIλ^+ (pMR10) was prepared by SDS alkaline lysis method (Sambrook et al., Molecular Cloning 2nd Ed. Cold Spring Harbor Press, 1989). Reaction conditions were as follows: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1uM each primer, 2.5U Taq polymerase, per 100 µl reaction. Cycling conditions were 94°C 2 minutes, then 35 cycles of 94°C 30s, 55°C 30s, 72°C 3 minutes, followed by 72°C for 7 minutes.

Example 1 Formulation of the enzyme in Mn conditions

In the following "pre-mix" protocol, all the reagents are contained in two solutions; reagent mix A and reagent mix B.

Reagent Mix A

The following reagents were combined to make 10 ml of reagent mix A:

2.5 ml 1 M HEPPS N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid), pH 8.0

500 µl 1 M tartaric acid, pH 8.0

50,000 units FY7 DNA polymerase

1 unit *Thermoplasma acidophilum* inorganic pyrophosphatase

100 µl 100 mM dATP

100 µl 100 mM dTTP

100 µl 100 mM dCTP

500 µl 100 mM dITP

9.375 µl 100 µM C-7-propargylamino-4-rhodamine-6-G-ddATP

90 µl 100 µM C-5-propargylamino-4-rhodamine-X-ddCTP

6.75 µl 100 µM C-7-propargylamino-4-rhodamine-110-ddGTP

165 µl 100 µM C-5-propargylamino-4-tetramethylrhodamine-ddUTP

10 µl 50 mM EDTA

1 ml glycerol

The volume was made up to 10,000 µl with deionized H_2O.

Reagent Mix B

The following reagents were combined to make 10 ml of reagent mix B:

10 µl 1M MES 2-(N-morpholino)ethanesulfonic acid, pH 6.0

200 µl 1M $MgCl_2$

75 µl 1M $MnSO_4$

The volume was made up to 10,000 µl with deionized H_2O.

Example 2: Use of the formulation from Example 1

Two (2) µl reagent mix A, 2 µl reagent mix B, 200 ng M13mp18 DNA, 5 pmole of primer (M13 - 40 Forward 5'-GTTTTCCCAGTCACGACGTTGTA), and deionized water to a total volume of 20 µl were mixed together and subjected to 25 cycles of (95 °C 30 seconds, 60 °C 1 minute) in a thermal cycler. After cycling, 4 µl of a solution which contained 1.5 M sodium acetate, 250 mM EDTA was added. The solution was mixed and 4 volumes (100 µl) of ethanol added. The DNA was precipitated by incubation on ice for 15-20 minutes followed by centrifugation. The supernatant was removed and the pellet was washed with 70% ethanol, dried and resuspended in 4 µl of formamide containing loading dye. The resuspended DNA was then run on an automated fluorescent DNA sequencing apparatus (ABI model 377 instrument). The print out from the machine of the DNA sequence is shown as Figure 2.

Example 3 Formulation of the enzyme in Mg conditions

In the following "pre-mix" protocol, all the reagents are contained in one solution.

Sequencing premix

The following reagents were combined to make 800 µl of Sequencing premix

200 µl of 500 mM Tris-HCl pH 9.5, 20 mM $MgCl_2$

100 µl 40 units/µl FY7 DNA polymerase, 0.0008 units/µl *Thermoplasma acidophilum* inorganic pyrophosphatase

100 µl 10 mM dITP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP

100 µl 0.125 µM C-7-propargylamino-4-rhodamine-6-G-ddATP

100 µl 1.2 µM C-5-propargylamino-4-rhodamine-X-ddCTP

100 µl 0.09 µM C-7-propargylamino-4-rhodamine-110-ddGTP

100 µl 2.2 µM C-5-propargylamino-4-tetramethylrhodamine-ddUTP

Example 4 Use of the formulation from example 3

Four (4) µl of sequencing premix, 200 ng M13mp18 DNA, 5 pmole of primer (M13 -40 Forward 5'- GTTTTCCCAGTCACGACGTTGTA), and deionized water to a total volume of 20 µl were mixed together and subjected to 25 cycles of (95 °C 30 seconds, 60 °C 2 minutes) in a thermal cycler. After cycling, 7 µl of 7.5 M ammonium acetate was added. The solution was mixed and 4 volumes (100 µl) of ethanol added. The DNA was precipitated by incubation on ice for 15-20 minutes followed by centrifugation. The supernatant was removed and the pellet was washed with 70% ethanol, dried and resuspended in 4 µl of formamide containing loading dye. The resuspended DNA was then run on an automated fluorescent DNA sequencing apparatus (ABI model 377 instrument). The print out from the machine of the DNA sequence is shown as Figure 3.

Example 5 Polymerase Activity versus Salt Concentration (KCl) for Thermo Sequenase™ enzyme and FY7 enzyme.

The percent of maximum polymerase activity was measured for Thermo Sequenase™ enzyme DNA polymerase and FY7 DNA polymerase under varying KCl concentrations. The results are depicted in Figure 4. The data indicate that FY7 has a much higher salt optimum as well as broader range of tolerance for salt in the reaction mixture than Thermo Sequenase™. The salt concentration which gives 50% activity is five-fold higher for FY7 than for Thermo Sequenase.

The effect of high salt concentrations on DNA sequencing ability in radioactively labeled DNA sequencing reactions was also examined. The results are presented in Figure 5. At KCl concentrations of 50mM or higher Thermo Sequenase™ polymerase performance degrades to levels at which usable data cannot be extracted. FY7 DNA polymerase, however, is able to give quite good sequencing data at concentrations of KCl of 100mM.

Example 6 Fluorescent Sequencing Salt Tolerance

These experiments examined the effect of the above-demonstrated polymerase activity in high salt concentrations on DNA sequencing ability in fluorescently labeled terminator DNA sequencing reactions. The results are presented in Figures 6-15.

Figures 6-10 show the effect of increasing salt concentration on the performance of Thermo Sequenase. At concentrations as low as 25mM data quality is affected with the read length being decreased from at least 600 bases to about 450 bases. At 50mM salt the read length is further decreased to about 350 bases, 75mM to about 250 bases and at 100mM the read length is negligible.

Figures 11-15 show the effect of increasing salt concentration on the performance of FY7 DNA polymerase. There is no detrimental effect on performance to at least 75mM KCl and only a slight decrease in data quality at 100mM KCl.

As it is recognized that some types of DNA preparations may be contaminated with salt (which is detrimental to DNA sequencing data quality), the use of FY7 DNA polymerase allows for a more robust sequencing reaction over a broader range of template conditions.

Example 7 Polymerase Processivity

The processivity (number of nucleotides incorporated per DNA polymerase binding event) has been measured, for different DNA sequencing polymerases. The results are presented in Figure 16. Thermo Sequenase DNA polymerase has a processivity of only ~4 nucleotides per binding event. AmpliTaq FS DNA polymerase has a processivity of ~15 nucleotides per binding event. FY7 DNA polymerase has a processivity more than seven-fold greater than Thermo Sequenase DNA polymerase and ~two-fold greater than AmpliTaq FS DNA polymerase at ~30 nucleotides per binding event.

Example 8 Polymerase Extension with dITP at 72 °C

The series examined improved read length obtained when using FY7 polymerase versus Thermo Sequenase DNA polymerase in radioactively labeled sequencing reactions incorporating the dGTP (Guanosine triphosphate) analog dITP (Inosine triphosphate) at 72 °C. The results are presented in Figure 17. FY7 is able to incorporate >50-100 more nucleotides under standard $^{33}P[\alpha\text{-dATP}]$ sequencing conditions than Thermo Sequenase.

Example 9 Effect of Extension Step Time on Length of Read

These series of experiments examined the effect of increasing extension step time of the read length and data quality of Thermo Sequenase and FY7 DNA polymerases in fluorescently labeled terminator DNA sequencing reactions. The results are presented in Figures 18-27.

Figures 18-22 show the effect of increasing extension step time on the read length and data quality produced by Thermo Sequenase DNA polymerase. This data shows that a minimum of a two

minutes extension step is required by Thermo Sequenase in order to achieve a quality read of at least 600 bases. Signal strength generally increases to a maximum at a four minute extension (the time specified in the commercial product utilizing this enzyme and method).

Figures 23-27 show the effect of increasing extension step time on the read length and data quality produced by FY7 DNA polymerase. This data shows that a minimum of a 30 second extension step is required by FY7 in order to achieve a quality read of at least 600 bases. Signal strengths plateau at about one minute extension time. The FY7 DNA polymerase can produce data of equivalent quality to Thermo Sequenase in one-quarter to one-half the time of extension reaction.

Although the above examples describe various embodiments of the invention in detail, many variations will be apparent to those of ordinary skill in the art. Accordingly, the above examples are intended for illustration purposes and should not be used in any way to restrict the scope of the appended claims.

CLAIMS

What is claimed is:

1. A purified recombinant thermostable DNA polymerase comprising the amino acid sequence set forth in Figure 1.

2. A purified recombinant thermostable DNA polymerase which exhibits at least about 80% activity at salt concentations of 50 mM and greater.

3. A purified recombinant thermostable DNA polymerase which exhibits at least about 70% activity at salt concentrations of 25 mM and greater.

4. A purified recombinant thermostable DNA polymerase having a processivity of about 30 nucleotides per binding event.

5. An isolated nucleic acid that encodes a thermostable DNA polymerase, wherein said nucleic acid consists of the nucleotide sequence set forth in Figure 1

6. A recombinant DNA vector that comprises the nucleic acid of Claim 3.

7. The recombinant DNA sequence of Claim 4 comprising the plasmid pMR10.

8. A recombinant host cell transformed with the vector of Claim 5.

9. The recombinant host cell of Claim 6 that is *E. coli.*

10. The recombinant host cell of Claim 7 which is E. coli carrying the cI^+ and cI857 alleles.

11. The recombinant host cell of Claim 7 selected from the group consisting of DHIλ^+ [gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44, λ^-] and M5248 [λ (bio275, cI857, cIII+, N+, λ (H1))].

12. Method of sequencing DNA comprising the step of generating chain terminated fragments from the DNA template to be sequenced with the DNA polymerase of Claim 1 in the presence of at least one chain terminating agent and one or more nucleotide triphosphates, and determining the sequence of said DNA from the sizes of said fragments.

13. A kit for sequencing DNA comprising the DNA polymerase of Claim 1.

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                                                                  31/11
/1
ATG GAA GCG ATG CTG CCG CTG TTC GAA CCC AAA GGC CGT GTC CTC CTG GTG GCC GGC CAC
M   E   A   M   L   P   L   F   E   P   K   G   R   V   L   L   V   A   G   H
                                                                  91/31
61/21
CAC CTG GCC TAC CGC ACC TTC TTC GCC CTG AAG GGC CTC ACC ACG AGC CGG GGC GAA CCG
H   L   A   Y   R   T   F   F   A   L   K   G   L   T   T   S   R   G   E   P
                                                                  151/51
121/41
GTG CAG GCG GTC TAC GGC TTC GCC AAG AGC CTC CTC AAG GCC CTG AAG GAG GAC GGG TAC
V   Q   A   V   Y   G   F   A   K   S   L   L   K   A   L   K   E   D   G   Y
                                                                  211/71
181/61
AAG GCC GTC TTC GTG GTC TTT GAC GCC AAG GCC CCC TCC TTC CGC CAC GAG GCC TAC GAG
K   A   V   F   V   V   F   D   A   K   A   P   S   F   R   H   E   A   Y   E
                                                                  271/91
241/81
GCC TAC AAG GCG GGG AGG GCC CCG ACC CCC GAG GAC TTC CCC CGG CAG CTC GCC CTC ATC
A   Y   K   A   G   R   A   P   T   P   E   D   F   P   R   Q   L   A   L   I
                                                                  331/111
301/101
AAG GAG CTG GTG GAC CTC CTG GGG TTT ACC CGC CTC GAG GTC CCC GGC TAC GAG GCG GAC
K   E   L   V   D   L   L   G   F   T   R   L   E   V   P   G   Y   E   A   D
                                                                  391/131
361/121
GAC GTT CTC GCC ACC CTG GCC AAG AAG GCG GAA AAG GAG GGG TAC GAG GTG CGC ATC CTC
D   V   L   A   T   L   A   K   K   A   E   K   E   G   Y   E   V   R   I   L
                                                                  451/151
421/141
ACC GCC GAC CGC GAC CTC TAC CAA CTC GTC TCC GAC CGC GTC GCC GTC CTC CAC CCC GAG
T   A   D   R   D   L   Y   Q   L   V   S   D   R   V   A   V   L   H   P   E
                                                                  511/171
481/161
GGC CAC CTC ATC ACC CCG GAG TGG CTT TGG GAG AAG TAC GGC CTC AGG CCG GAG CAG TGG
G   H   L   I   T   P   E   W   L   W   E   K   Y   G   L   R   P   E   Q   W
                                                                  571/191
541/181
GTG GAC TTC CGC GCC CTC GTG GGG GAC CCC TCC GAC AAC CTC CCC GGG GTC AAG GGC ATC
V   D   F   R   A   L   V   G   D   P   S   D   N   L   P   G   V   K   G   I
                                                                  631/211
601/201
GGG GAG AAG ACC GCC CTC AAG CTC CTC AAG GAG TGG GGA AGC CTG GAA AAC CTC CTC AAG
G   E   K   T   A   L   K   L   L   K   E   W   G   S   L   E   N   L   L   K
                                                                  691/231
661/221
AAC CTG GAC CGG GTA AAG CCA GAA AAC GTC CGG GAG AAG ATC AAG GCC CAC CTG GAA GAC
N   L   D   R   V   K   P   E   N   V   R   E   K   I   K   A   H   L   E   D
                                                                  751/251
721/241
CTC AGG CTC TCC TTG GAG CTC TCC CGG GTG CGC ACC GAC CTC CCC CTG GAG GTG GAC CTC
L   R   L   S   L   E   L   S   R   V   R   T   D   L   P   L   E   V   D   L
                                                                  811/271
781/261
GCC CAG GGG CGG GAG CCC GAC CGG GAG GGG CTT AGG GCC TTC CTG GAG AGG CTG GAA TTC
A   Q   G   R   E   P   D   R   E   G   L   R   A   F   L   E   R   L   E   F
                                                                  871/291
841/281
GGC AGC CTC CTC CAC GAG TTC GGC CTC CTG GAG GCC CCC GCC CCC CTG GAG GAG GCC CCC
G   S   L   L   H   E   F   G   L   L   E   A   P   A   P   L   E   E   A   P
                                                                  931/311
901/301
TGG CCC CCG CCG GAA GGG GCC TTC GTG GGC TTC GTC CTC TCC CGC CCC GAG CCC ATG TGG
W   P   P   P   E   G   A   F   V   G   F   V   L   S   R   P   E   P   M   W
                                                                  991/331
961/321
GCG GAG CTT AAA GCC CTG GCC GCC TGC AGG GAC GGC CGG GTG CAC CGG GCA GCA GAC CCC
A   E   L   K   A   L   A   A   C   R   D   G   R   V   H   R   A   A   D   P
                                                                  1051/351
1021/341
TTG GCG GGG CTA AAG GAC CTC AAG GAG GTC CGG GGC CTC CTC GCC AAG GAC CTC GCC GTC
L   A   G   L   K   D   L   K   E   V   R   G   L   L   A   K   D   L   A   V
                                                                  1111/371
1081/361
TTG GCC TCG AGG GAG GGG CTA GAC CTC GTG CCC GGG GAC GAC CCC ATG CTC CTC GCC TAC
L   A   S   R   E   G   L   D   L   V   P   G   D   D   P   M   L   L   A   Y
                                                                  1171/391
1141/381
CTC CTG GAC CCC TCC AAC ACC ACC CCC GAG GGG GTG GCG CGG CGC TAC GGG GGG GAG TGG
L   L   D   P   S   N   T   T   P   E   G   V   A   R   R   Y   G   G   E   W
                                                                  1231/411
1201/401
```

Figure 1

```
ACG GAG GAC GCC GCC CAC CGG GCC CTC CTC TCG GAG AGG CTC CAT CGG AAC CTC CTT AAG
T   E   D   A   A   H   R   A   L   L   S   E   R   L   H   R   N   L   L   K
1261/421                                                1291/431
CGC CTC GAG GGG GAG GAG AAG CTC CTT TGG CTC TAC CAC GAG GTG GAA AAG CCC CTC TCC
R   L   E   G   E   E   K   L   L   W   L   Y   H   E   V   E   K   P   L   S
1321/441                                                1351/451
CGG GTC CTG GCC CAC ATG GAG GCC ACC GGG GTA CGG CTG GAC GTG GCC TAC CTT CAG GCC
R   V   L   A   H   M   E   A   T   G   V   R   L   D   V   A   Y   L   Q   A
1381/461                                                1411/471
CTT TCC CTG GAG CTT GCG GAG GAG ATC CGC CGC CTC GAG GAG GAG GTC TTC CGC TTG GCG
L   S   L   E   L   A   E   E   I   R   R   L   E   E   E   V   F   R   L   A
1441/481                                                1471/491
GGC CAC CCC TTC AAC CTC AAC TCC CGG GAC CAG CTG GAA AGG GTG CTC TTT GAC GAG CTT
G   H   P   F   N   L   N   S   R   D   Q   L   E   R   V   L   F   D   E   L
1501/501                                                1531/511
AGG CTT CCC GCC TTG GGG AAG ACG CAA AAG ACA GGC AAG CGC TCC ACC AGC GCC GCG GTG
R   L   P   A   L   G   K   T   Q   K   T   G   K   R   S   T   S   A   A   V
1561/521                                                1591/531
CTG GAG GCC CTA CGG GAG GCC CAC CCC ATC GTG GAG AAG ATC CTC CAG CAC CGG GAG CTC
L   E   A   L   R   E   A   H   P   I   V   E   K   I   L   Q   H   R   E   L
1621/541                                                1651/551
ACC AAG CTC AAG AAC ACC TAC GTG GAC CCC CTC CCA AGC CTC GTC CAC CCG AGG ACG GGC
T   K   L   K   N   T   Y   V   D   P   L   P   S   L   V   H   P   R   T   G
1681/561                                                1711/571
CGC CTC CAC ACC CGC TTC AAC CAG ACG GCC ACG GCC ACG GGG AGG CTT AGT AGC TCC GAC
R   L   H   T   R   F   N   Q   T   A   T   A   T   G   R   L   S   S   S   D
1741/581                                                1771/591
CCC AAC CTG CAG AAC ATC CCC GTC CGC ACC CCC TTG GGC CAG AGG ATC CGC CGG GCC TTC
P   N   L   Q   N   I   P   V   R   T   P   L   G   Q   R   I   R   R   A   F
1801/601                                                1831/611
GTG GCC GAG GCG GGT TGG GCG TTG GTG GCC CTG GAC TAT AGC CAG ATA GAG CTC CGC GTC
V   A   E   A   G   W   A   L   V   A   L   D   Y   S   Q   I   E   L   R   V
1861/621                                                1891/631
CTC GCC CAC CTC TCC GGG GAC GAA AAC CTG ATC AGG GTC TTC CAG GAG GGG AAG GAC ATC
L   A   H   L   S   G   D   E   N   L   I   R   V   F   Q   E   G   K   D   I
1921/641                                                1951/651
CAC ACC CAG ACC GCA AGC TGG ATG TTC GGC GTC CCC CCG GAG GCC GTG GAC CCC CTG ATG
H   T   Q   T   A   S   W   M   F   G   V   P   P   E   A   V   D   P   L   M
1981/661                                                2011/671
CGC CGG GCG GCC AAG ACG GTG AAC TAC GGC GTC CTC TAC GGC ATG TCC GCC CAT AGG CTC
R   R   A   A   K   T   V   N   Y   G   V   L   Y   G   M   S   A   H   R   L
2041/681                                                2071/691
TCC CAG GAG CTA GCC ATC CCC TAC GAA GAA GCG GTG GCC TTT ATA GAG CGC TAC TTC CAA
S   Q   E   L   A   I   P   Y   E   E   A   V   A   F   I   E   R   Y   F   Q
2101/701                                                2131/711
AGC TTC CCC AAG GTG CGG GCC TGG ATA GAA AAG ACC CTG GAG GAG GGG AGG AAG CGG GGC
S   F   P   K   V   R   A   W   I   E   K   T   L   E   E   G   R   K   R   G
2161/721                                                2191/731
TAC GTG GAA ACC CTC TTC GGA AGA AGG CGC TAC GTG CCC GAC CTC AAC GCC CGG GTG AAG
Y   V   E   T   L   F   G   R   R   R   Y   V   P   D   L   N   A   R   V   K
2221/741                                                2251/751
AGC GTC AGG GAG GCC GCG GAG CGC ATG GCC TTC AAC ATG CCC GTC CAG GGC ACC GCC GCC
S   V   R   E   A   A   E   R   M   A   F   N   M   P   V   Q   G   T   A   A
2281/761                                                2311/771
GAC CTC ATG AAG CTC GCC ATG GTG AAG CTC TTC CCC CGC CTC CGG GAG ATG GGG GCC CGC
D   L   M   K   L   A   M   V   K   L   F   P   R   L   R   E   M   G   A   R
2341/781                                                2371/791
ATG CTC CTC CAG GTC CAC GAC GAG CTC CTC CTG GAG GCC CCC CAA GCG CGG GCC GAG GAG
M   L   L   Q   V   H   D   E   L   L   L   E   A   P   Q   A   R   A   E   E
2401/801                                                2431/811
GTG GCG GCT TTG GCC AAG GAG GCC ATG GAG AAG GCC TAT CCC CTC GCC GTG CCC CTG GAG
V   A   A   L   A   K   E   A   M   E   K   A   Y   P   L   A   V   P   L   E
```

Figure 1 (continued)

```
                                                                2491/831
2461/821
GTG GAG GTG GGG ATG GGG GAG GAC TGG CTT TCC GCC AAG GGT TAG
V   E   V   G   M   G   E   D   W   L   S   A   K   G   *
```

Figure 1 (continued)

Model 377
Version 3.0

34-02/26/98

FY7v2 M13 200ng 60C/1min
Lane 34

Signal G:455 A:555 T:938 C:515
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1069 to 8260 Base 1: 1069

Fri, Feb 27, 1998 9:10 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.06 ABI100

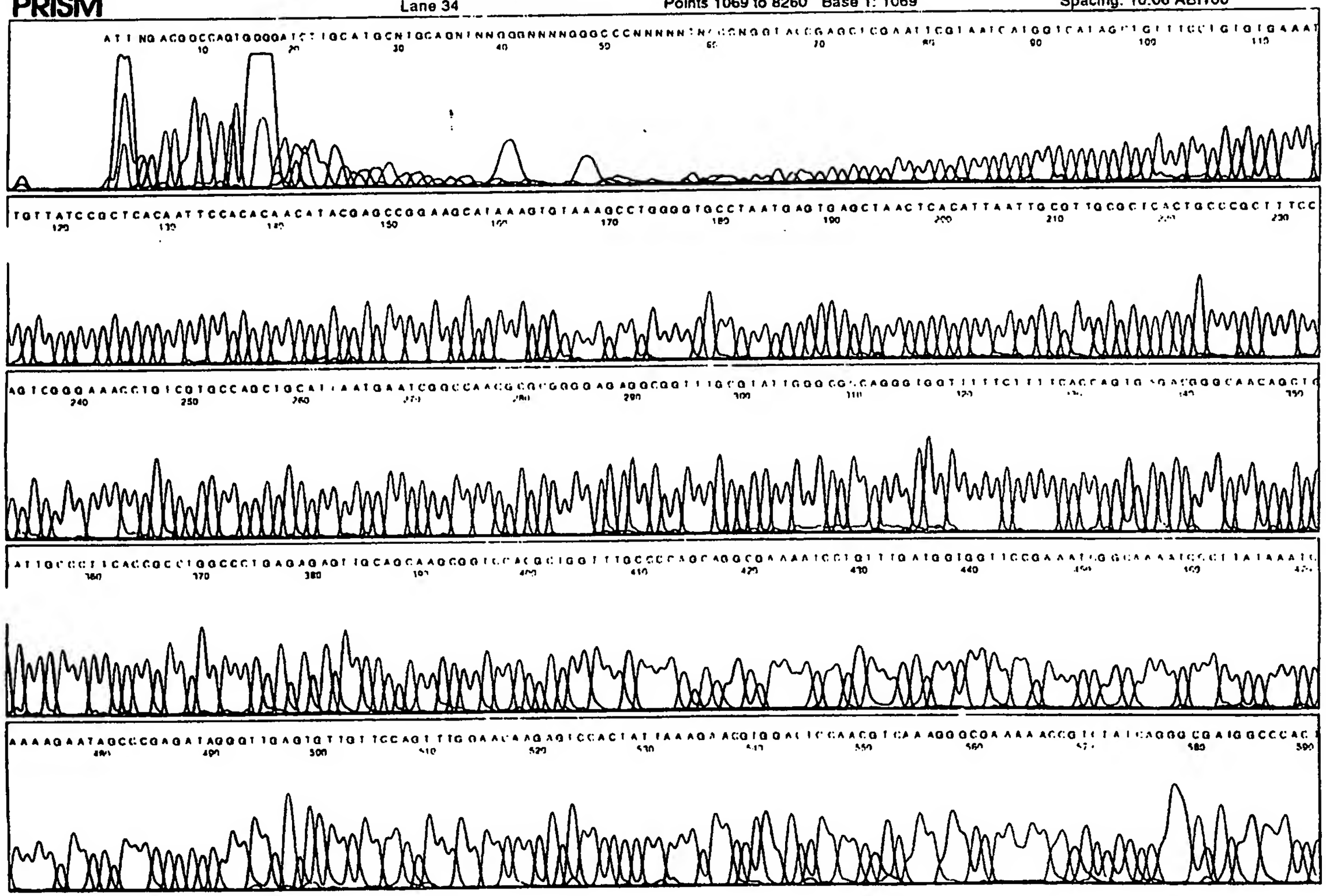

Figure 2

WO 99/65938
PCT/US99/13741

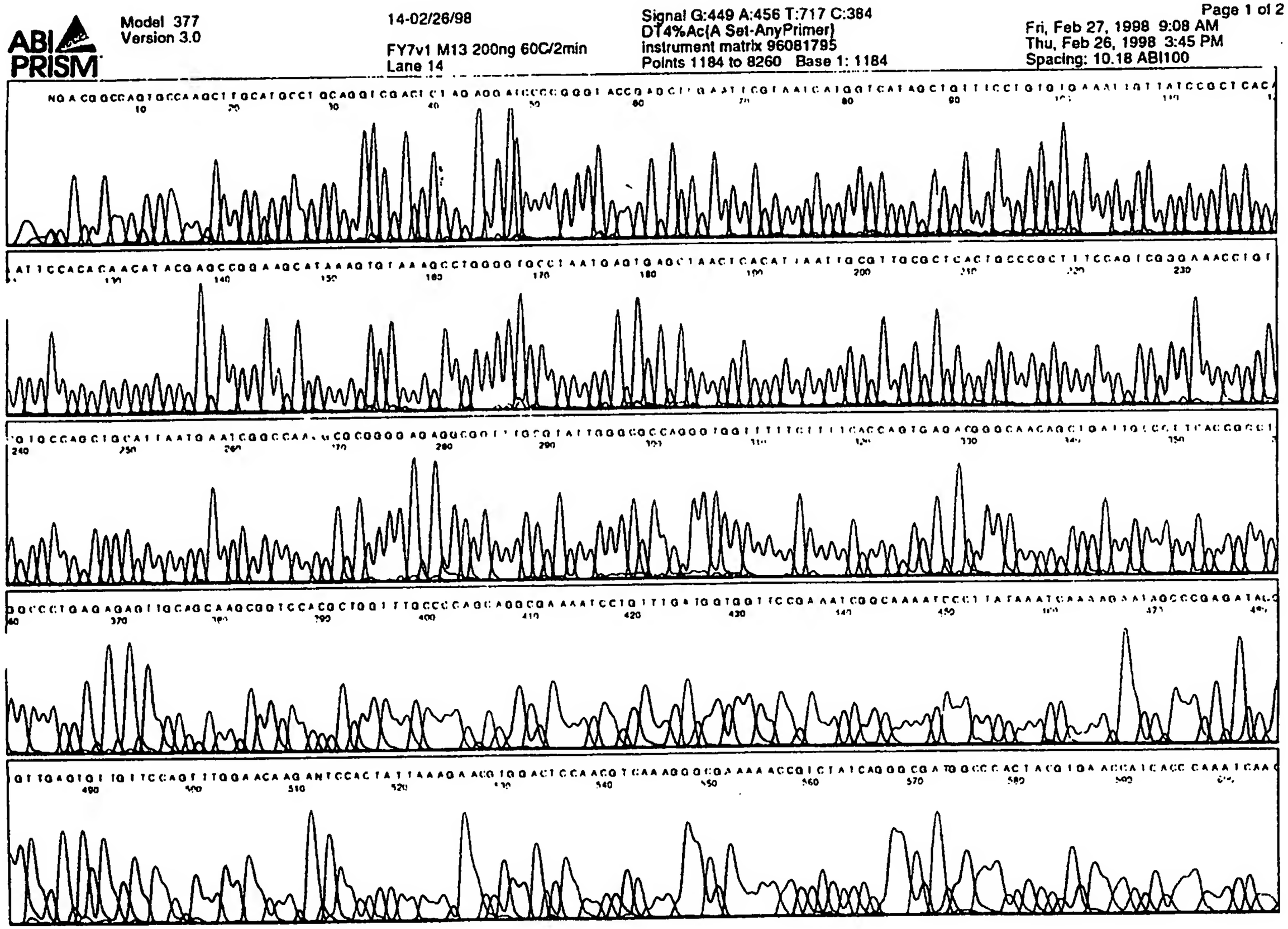
ABI PRISM
Model 377
Version 3.0
14-02/26/98
FY7v1 M13 200ng 60C/2min
Lane 14
Signal G:449 A:456 T:717 C:384
DT4%Ac(A Set-AnyPrimer)
instrument matrix 96081795
Points 1184 to 8260 Base 1: 1184
Page 1 of 2
Fri, Feb 27, 1998 9:08 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.18 ABI100

Figure 3

**Polymerase Activity versus Salt Concentration (KCl)
for Thermo Sequenase and FY7**

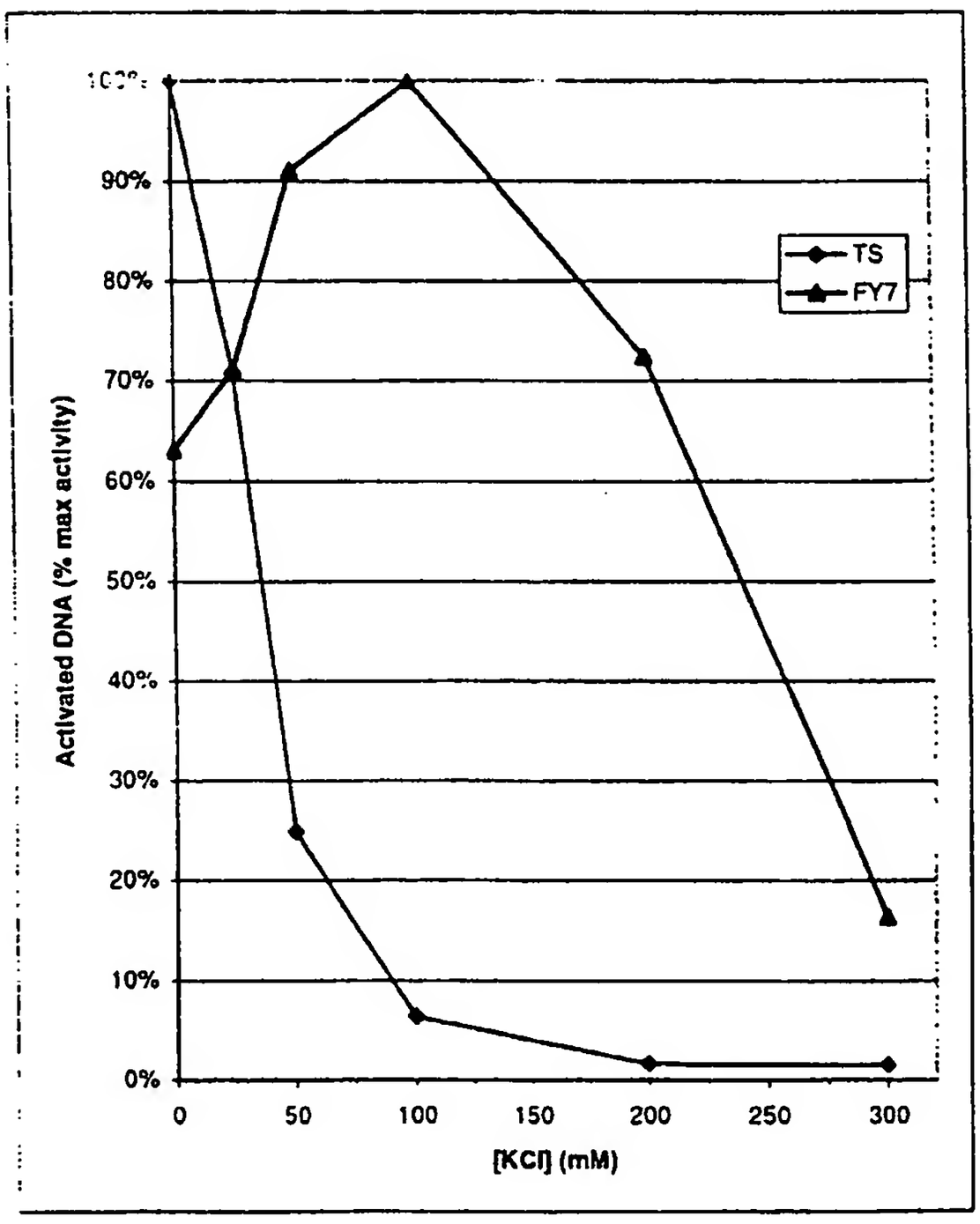

Figure 4

SALT TOLERANCE (KCl) OF FY7 VS. THERMO SEQUENASE

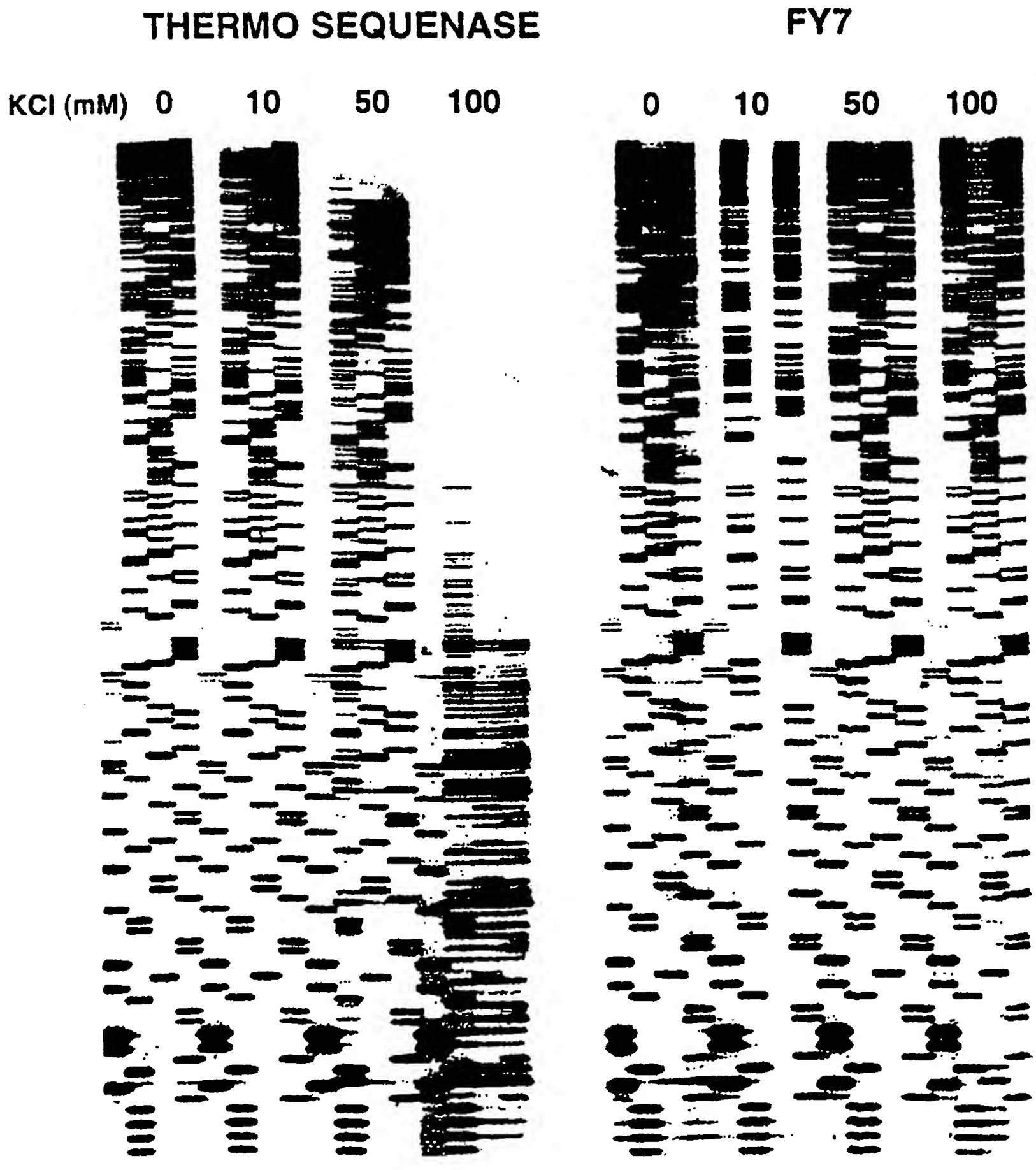

Figure 5

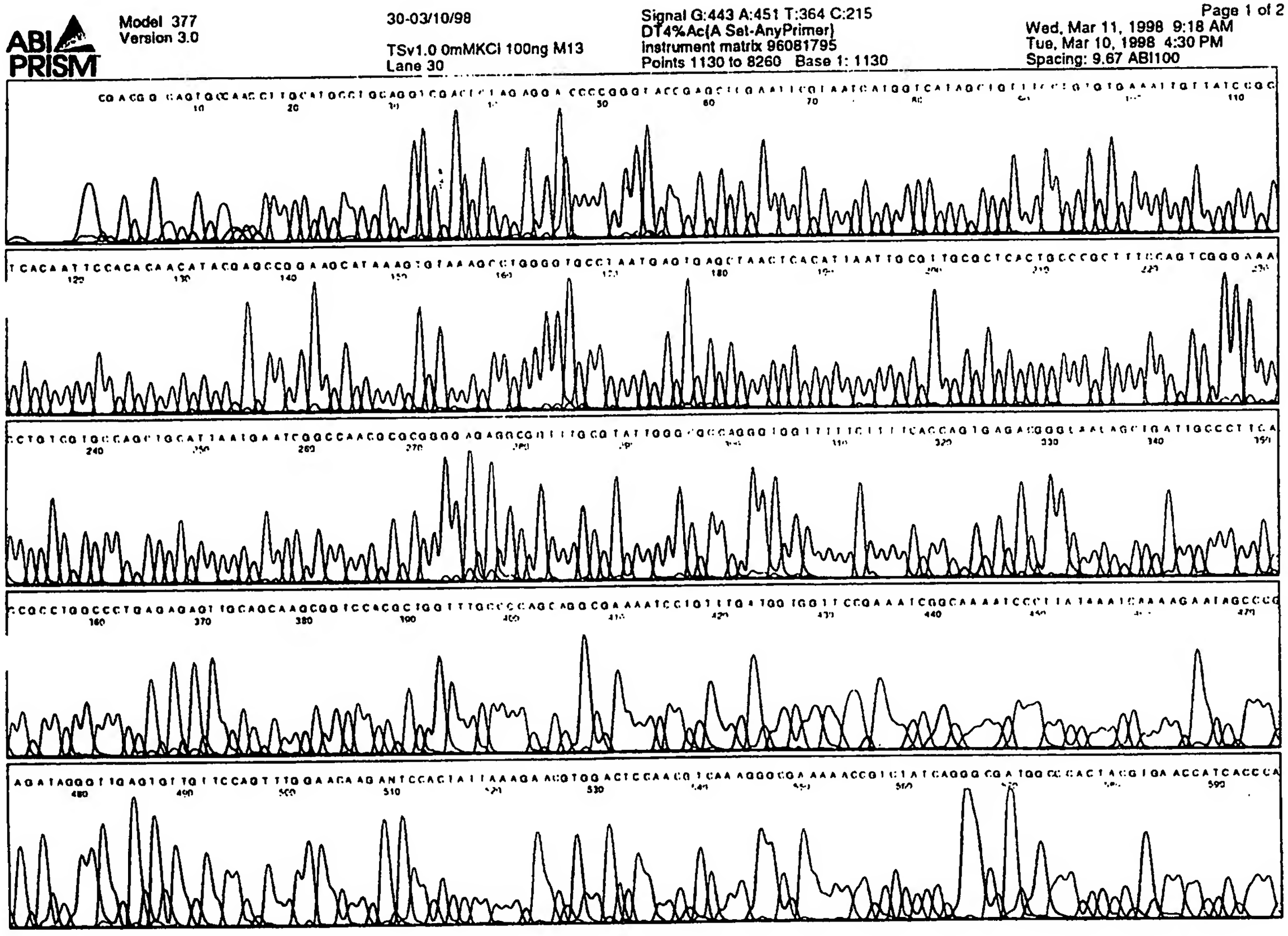
ABI PRISM
Model 377
Version 3.0
30-03/10/98
TSv1.0 0mMKCl 100ng M13
Lane 30
Signal G:443 A:451 T:364 C:215
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1130 to 8260 Base 1: 1130
Page 1 of 2
Wed, Mar 11, 1998 9:18 AM
Tue, Mar 10, 1998 4:30 PM
Spacing: 9.67 ABI100

Figure 6

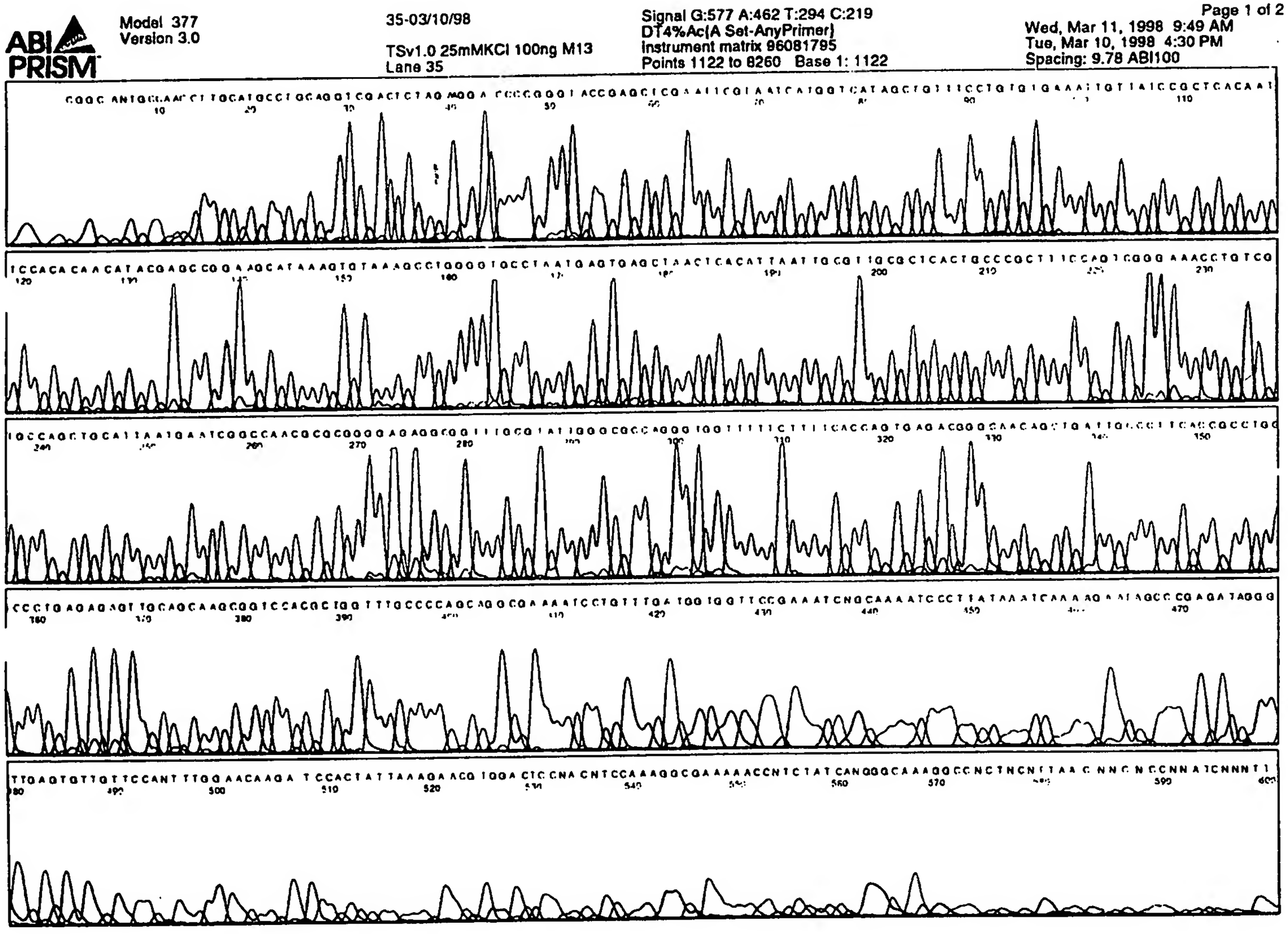
ABI PRISM
Model 377
Version 3.0
35-03/10/98
TSv1.0 25mMKCl 100ng M13
Lane 35
Signal G:577 A:462 T:294 C:219
DT4%Ac{A Set-AnyPrimer}
Instrument matrix 96081795
Points 1122 to 8260 Base 1: 1122
Page 1 of 2
Wed, Mar 11, 1998 9:49 AM
Tue, Mar 10, 1998 4:30 PM
Spacing: 9.78 ABI100

Figure 7

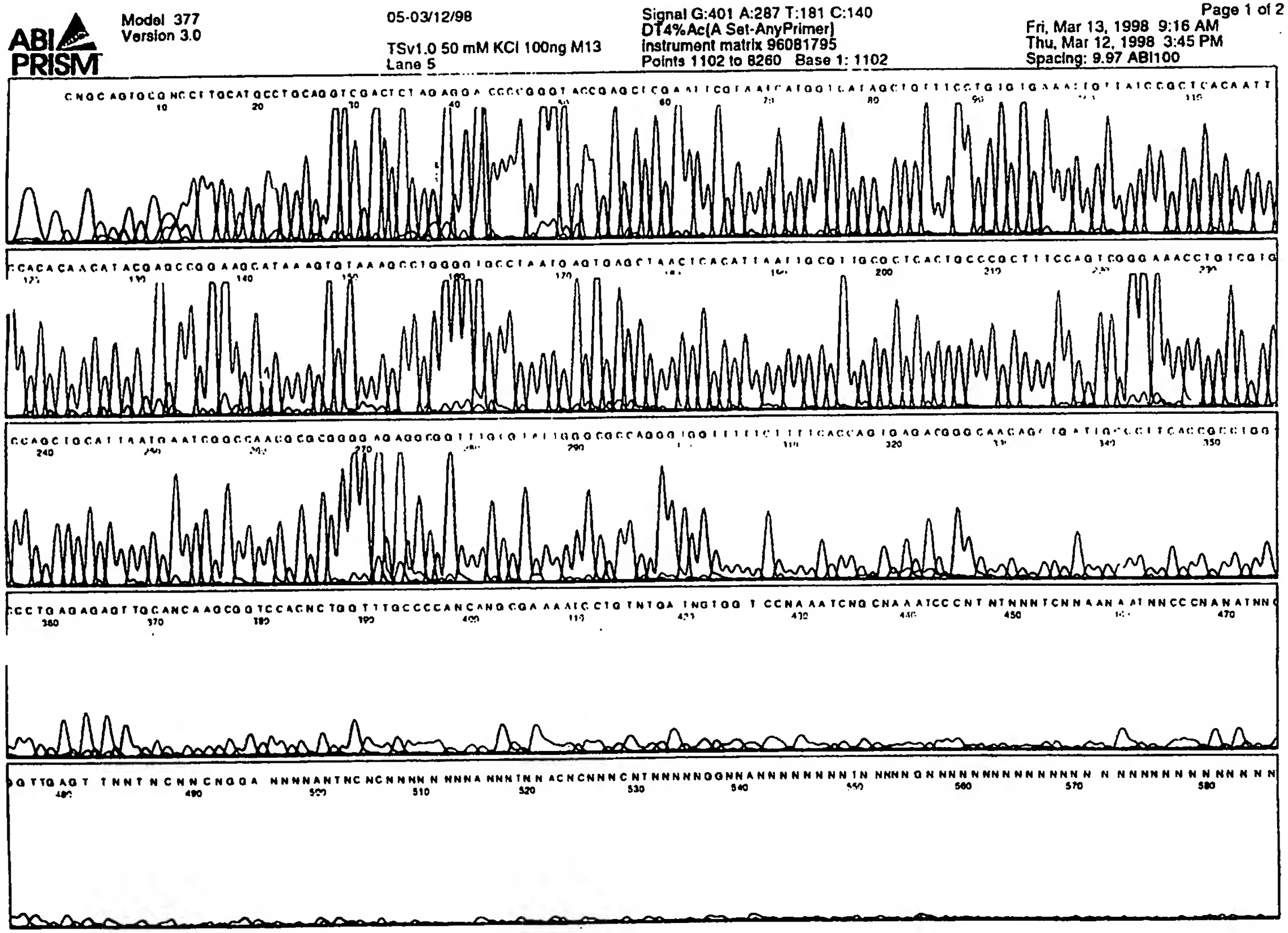
ABI PRISM
Model 377
Version 3.0
05-03/12/98
TSv1.0 50 mM KCl 100ng M13
Lane 5
Signal G:401 A:287 T:181 C:140
DT4%Ac(A Set-AnyPrimer)
instrument matrix 96081795
Points 1102 to 8260 Base 1: 1102
Page 1 of 2
Fri, Mar 13, 1998 9:16 AM
Thu, Mar 12, 1998 3:45 PM
Spacing: 9.97 ABI100

Figure 8

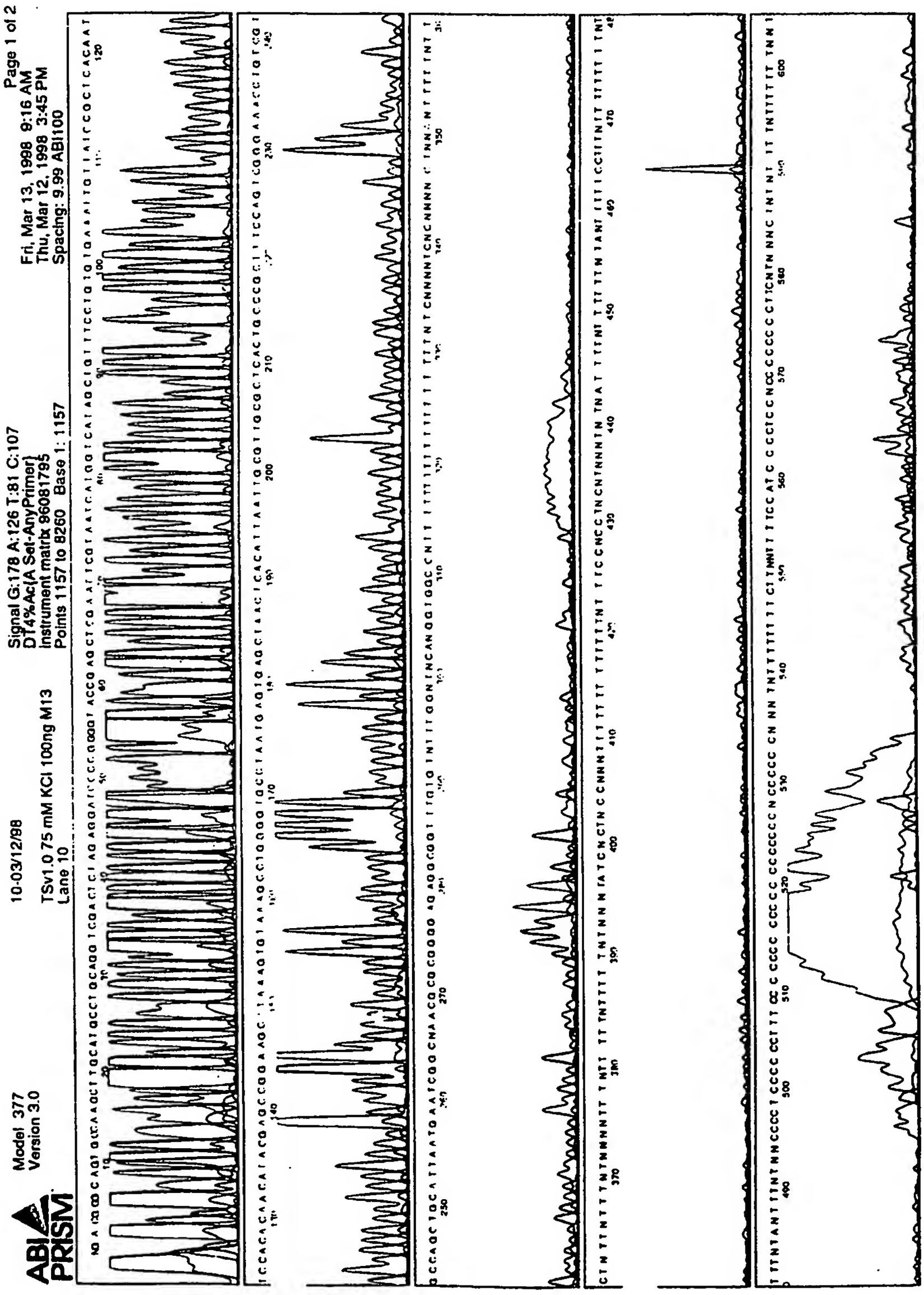
Page 1 of 2
Fri, Mar 13, 1998 9:16 AM
Thu, Mar 12, 1998 3:45 PM
Spacing: 9.99 ABI100
10-03/12/98
TSv1.0 75 mM KCl 100ng M13
Lane 10
Signal G:178 A:126 T:81 C:107
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1157 to 8260 Base 1: 1157
Model 377
Version 3.0
ABI PRISM

Figure 9

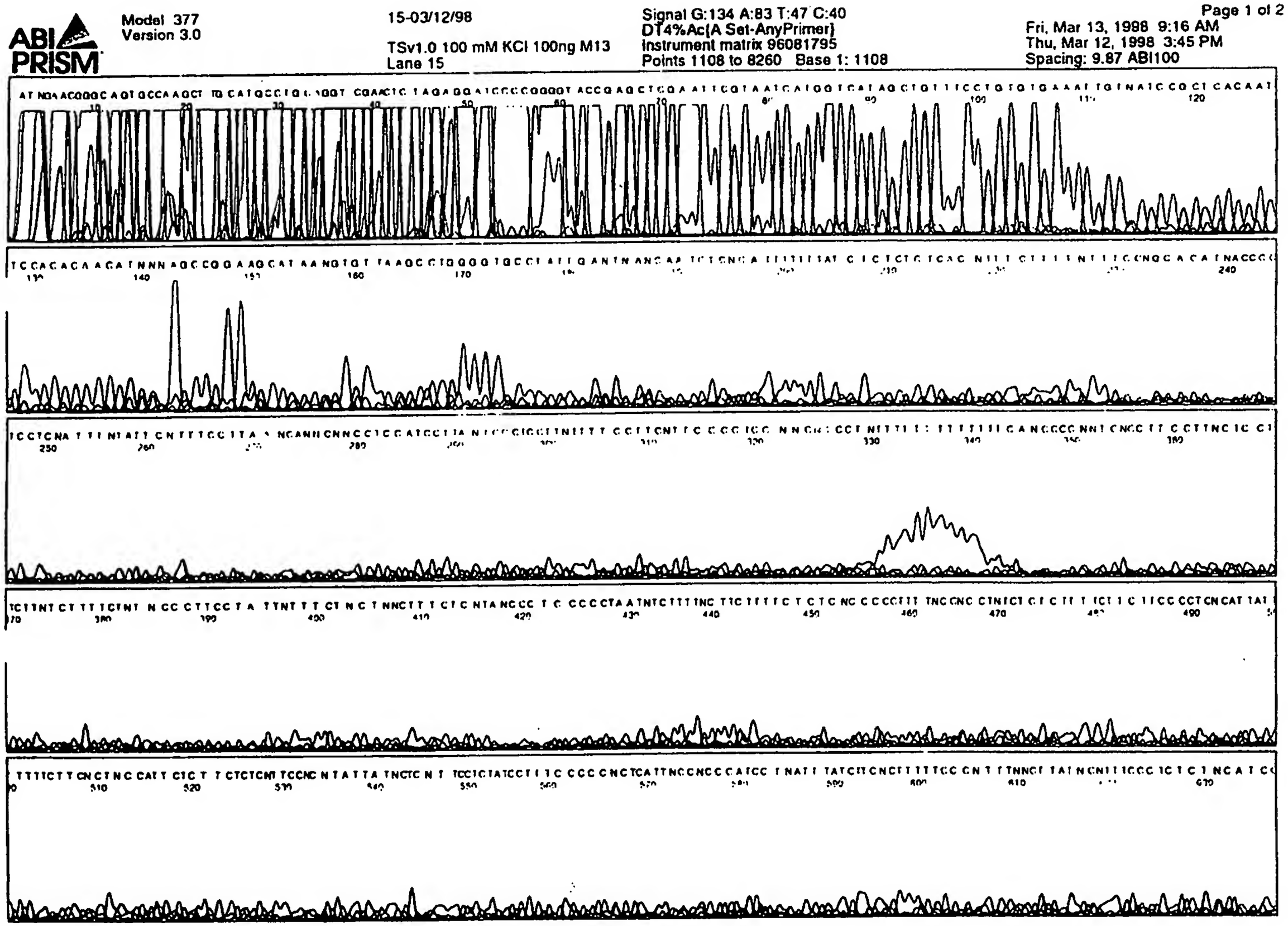
ABI PRISM
Model 377
Version 3.0
15-03/12/98
TSv1.0 100 mM KCl 100ng M13
Lane 15
Signal G:134 A:83 T:47 C:40
DT4%Ac{A Set-AnyPrimer}
Instrument matrix 96081795
Points 1108 to 8260 Base 1: 1108
Page 1 of 2
Fri, Mar 13, 1998 9:16 AM
Thu, Mar 12, 1998 3:45 PM
Spacing: 9.87 ABI100

Figure 10

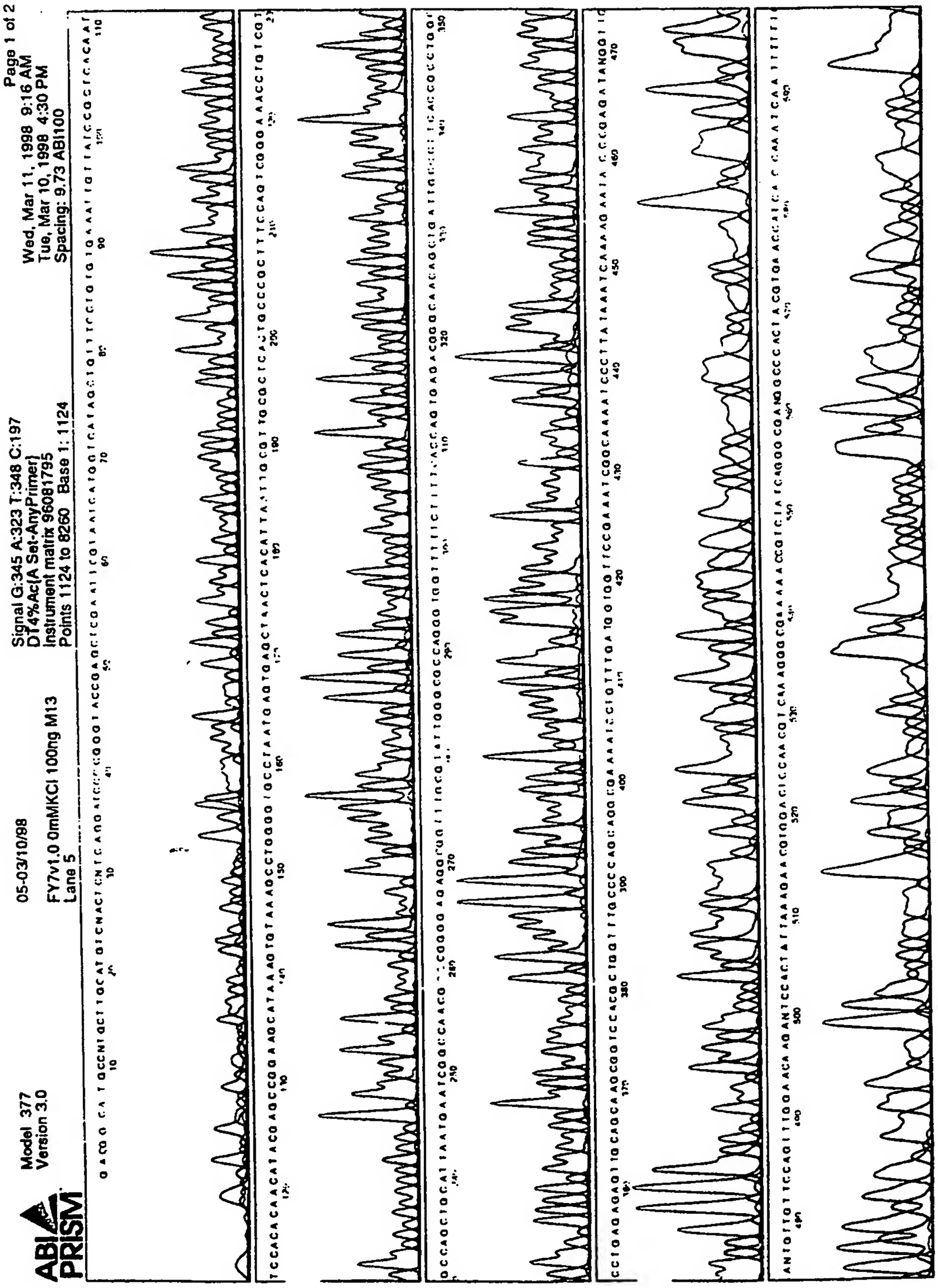
Page 1 of 2
Wed, Mar 11, 1998 9:16 AM
Tue, Mar 10, 1998 4:30 PM
Spacing: 9.73 ABI100
05-03/10/98
FY7v1.0 0mMKCl 100ng M13
Lane 5
Signal G:345 A:323 T:348 C:197
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1124 to 8260 Base 1: 1124
ABI PRISM
Model 377
Version 3.0

Figure 11

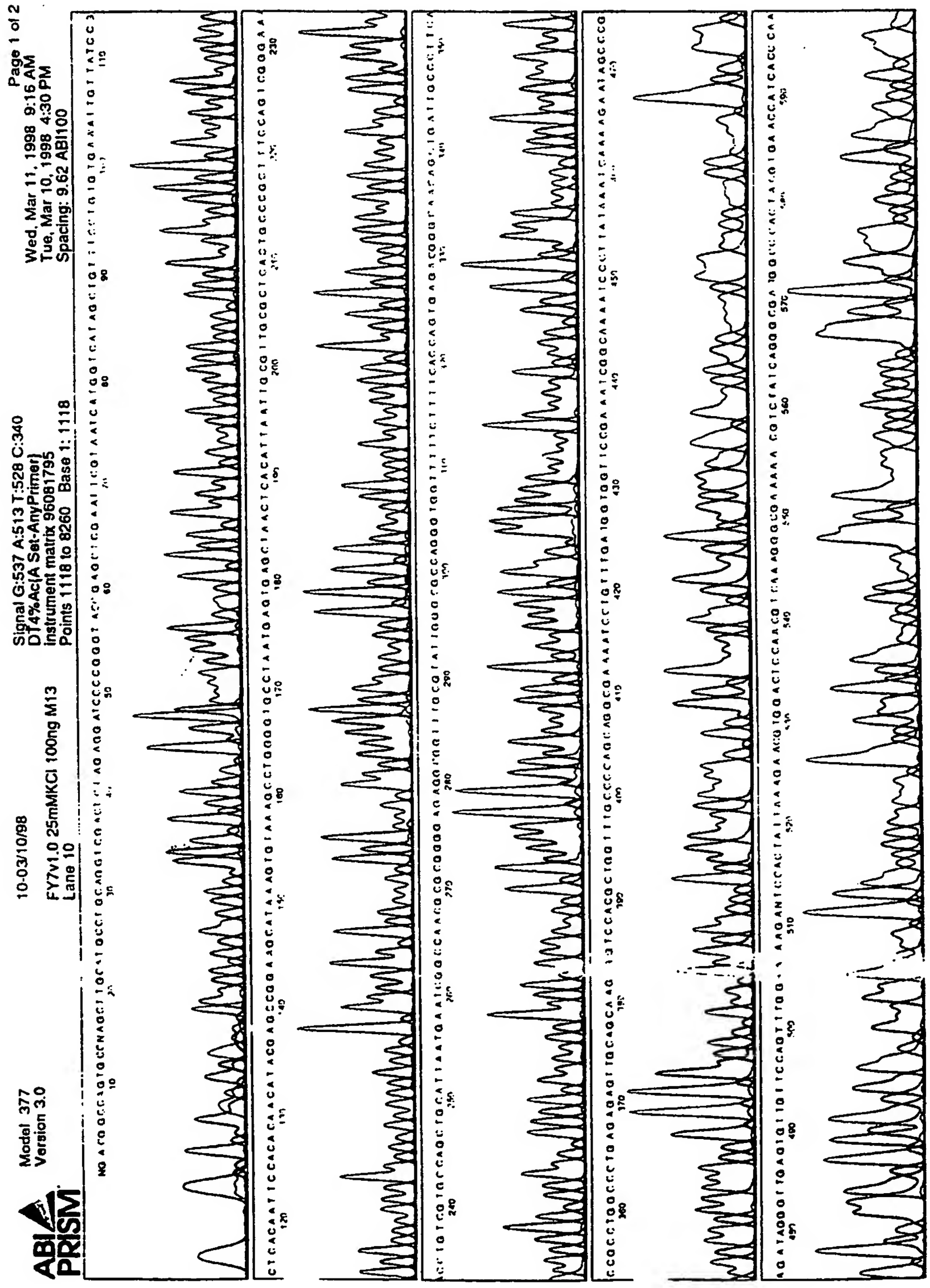
Page 1 of 2
Wed, Mar 11, 1998 9:16 AM
Tue, Mar 10, 1998 4:30 PM
Spacing: 9.62 ABI100
10-03/10/98
FY7v1.0 25mMKCl 100ng M13
Lane 10
Signal G:537 A:513 T:528 C:340
Df 4%Acl(A Set-AnyPrimer)
instrument matrix 96081795
Points 1118 to 8260 Base 1: 1118
ABI PRISM
Model 377
Version 3.0

Figure 12

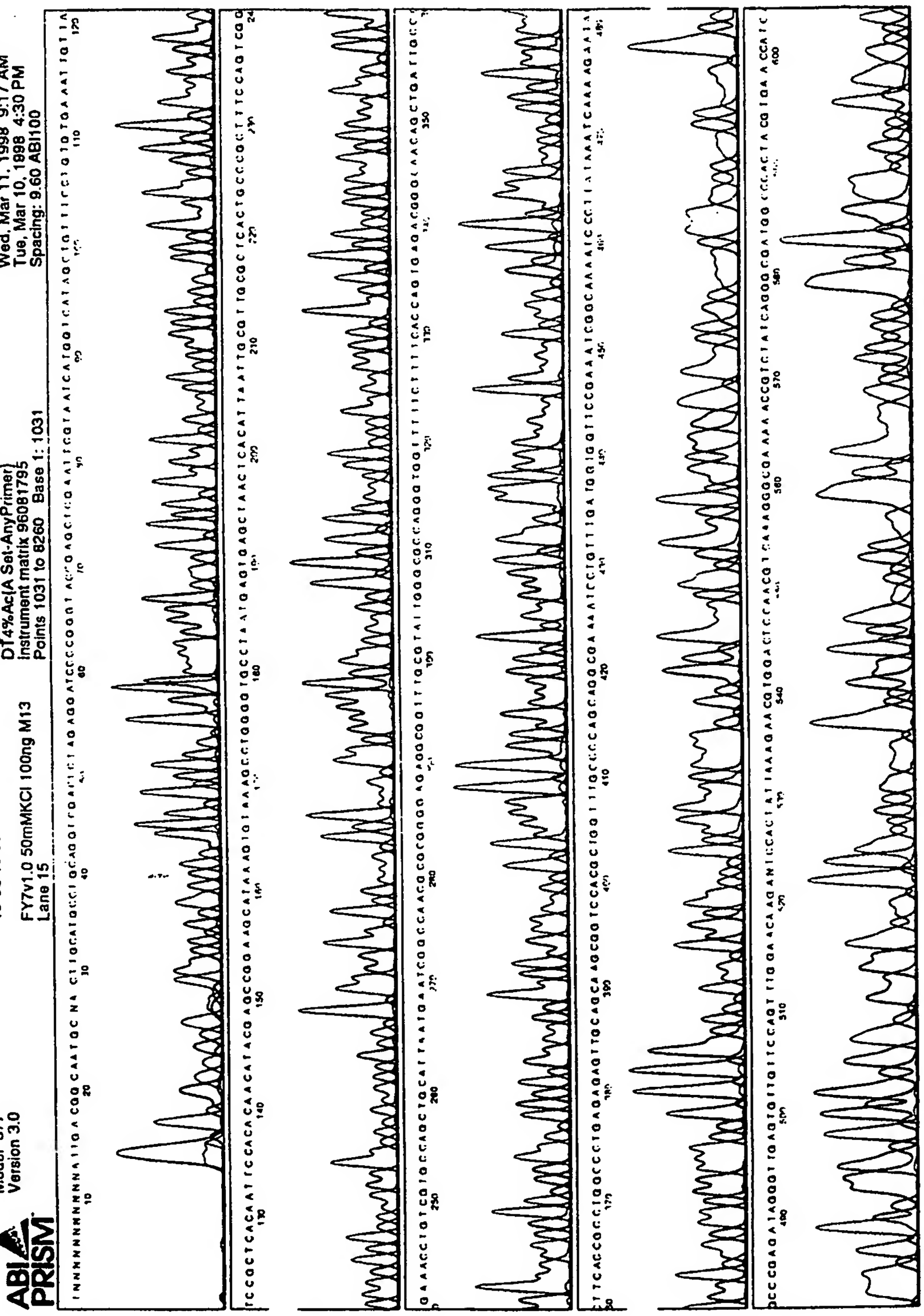
Page 1 of 2
Wed, Mar 11, 1998 9:17 AM
Tue, Mar 10, 1998 4:30 PM
Spacing: 9.60 ABI100
15-03/10/98
FY7v1.0 50mMKCl 100ng M13
Lane 15
Signal G:798 A:774 T:759 C:511
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1031 to 8260 Base 1: 1031
Model 377
Version 3.0
ABI PRISM

Figure 1.3

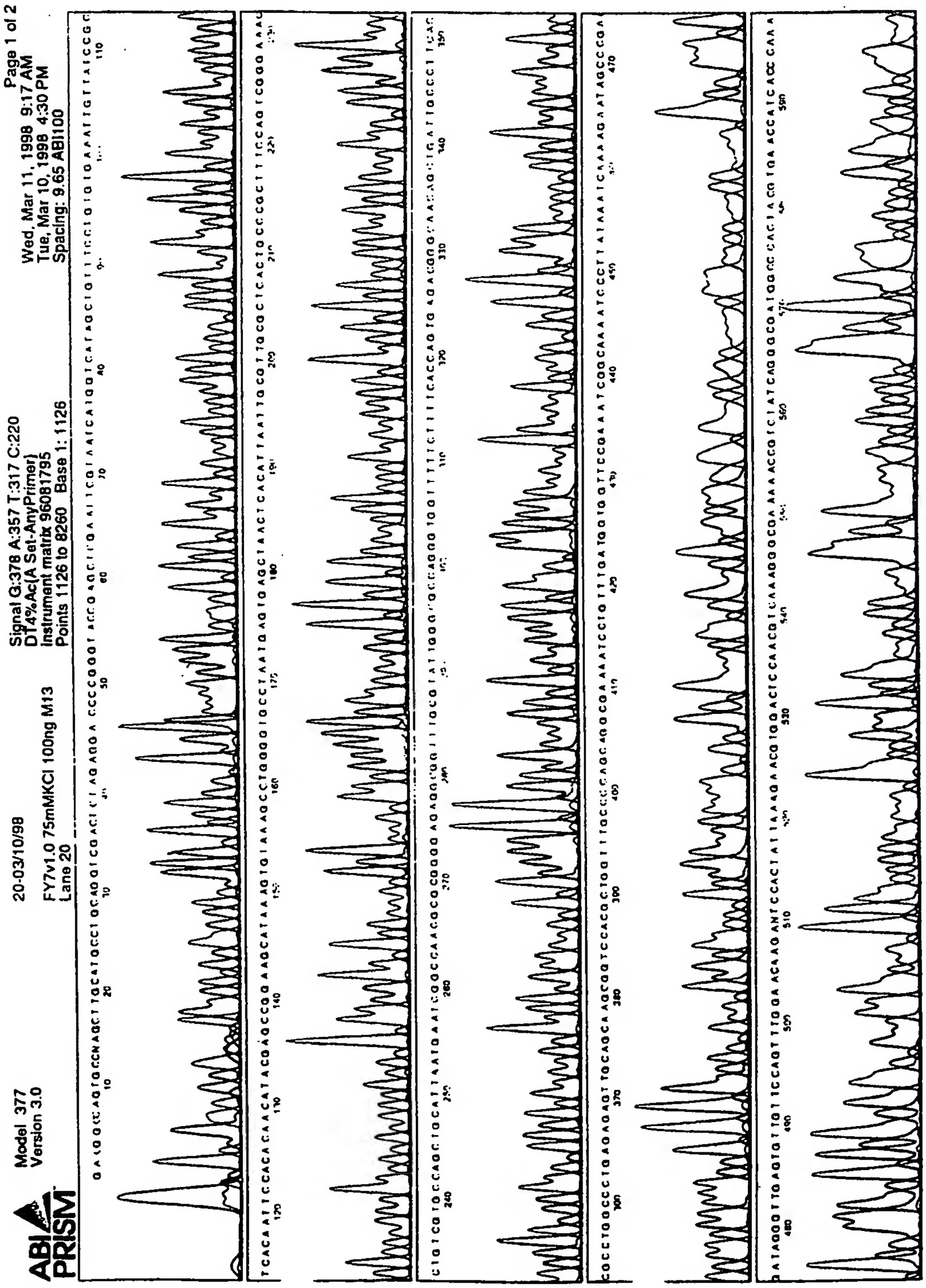
Page 1 of 2
Wed, Mar 11, 1998 9:17 AM
Tue, Mar 10, 1998 4:30 PM
Spacing: 9.65 ABI100
20-03/10/98
FY7v1.0 75mMKCl 100ng M13
Lane 20
Signal G:378 A:357 T:317 C:220
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1126 to 8260 Base 1: 1126
ABI PRISM
Model 377
Version 3.0

Figure 14

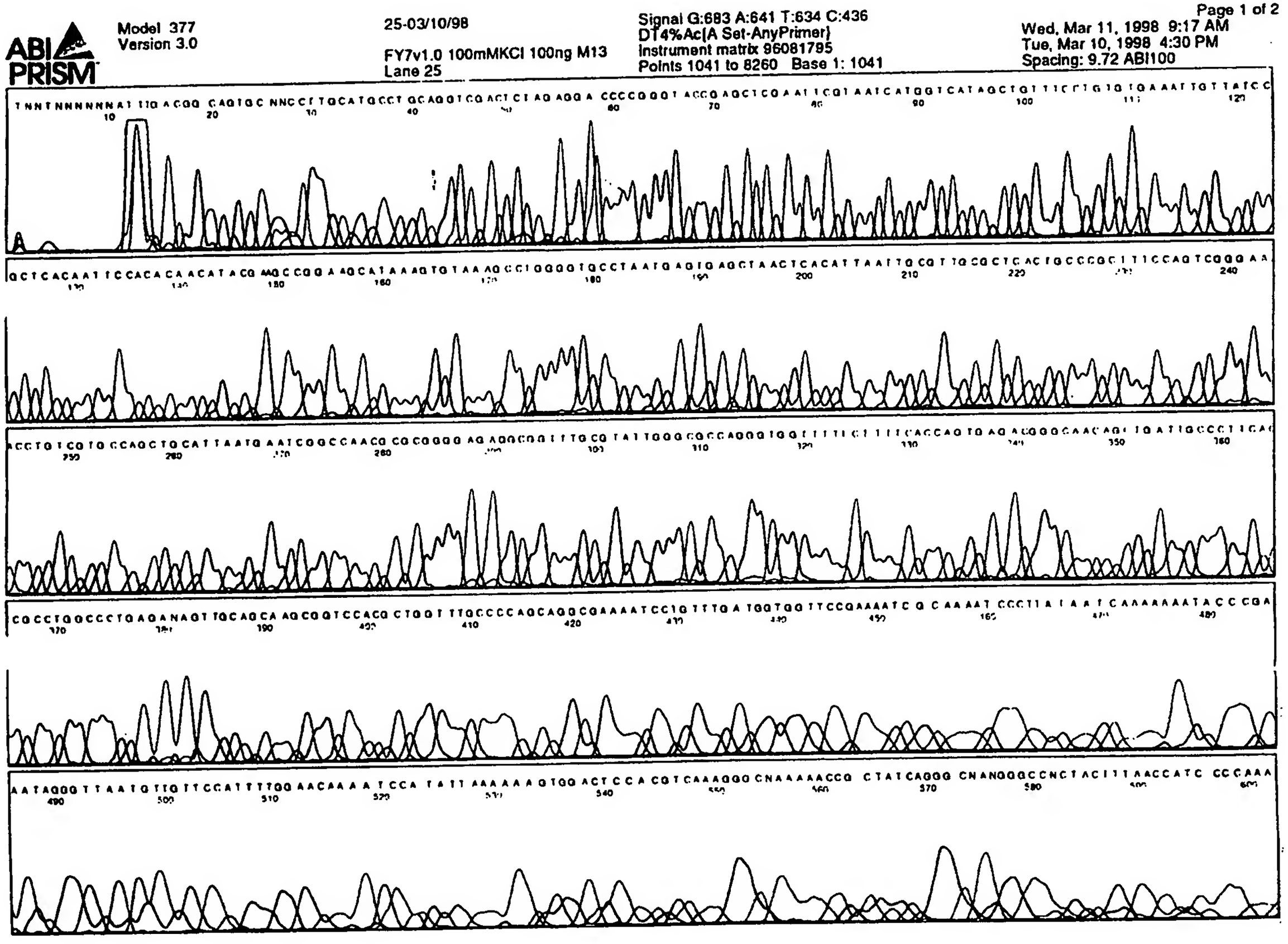
ABI PRISM
Model 377
Version 3.0
25-03/10/98
FY7v1.0 100mMKCl 100ng M13
Lane 25
Signal G:683 A:641 T:634 C:436
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1041 to 8260 Base 1: 1041
Page 1 of 2
Wed, Mar 11, 1998 9:17 AM
Tue, Mar 10, 1998 4:30 PM
Spacing: 9.72 ABI100

Figure 15

Polymerase	Processivity number of nucleotides
Thermo Sequenase	4
Amplitaq FS	15
FY7	30

Figure 16

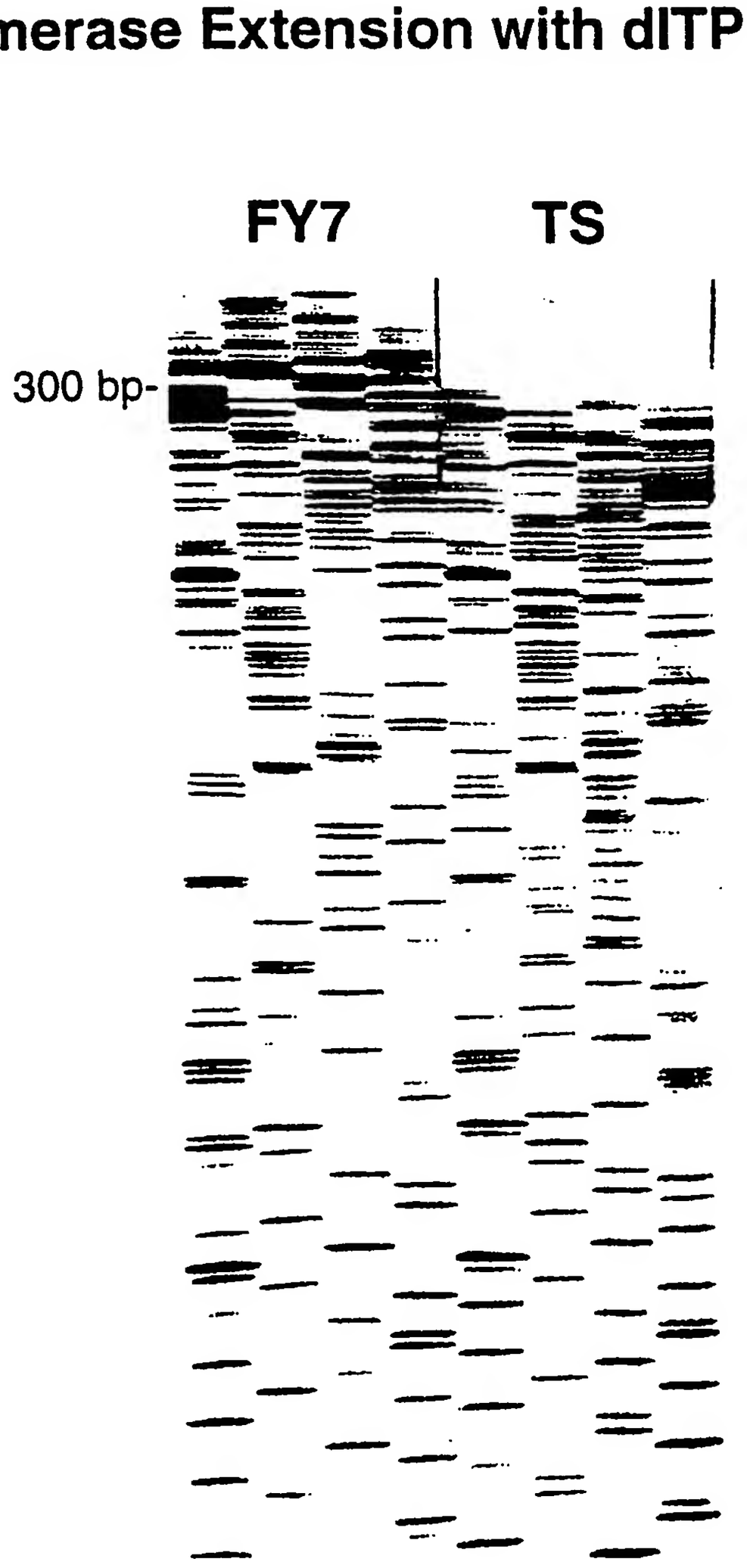

Figure 17

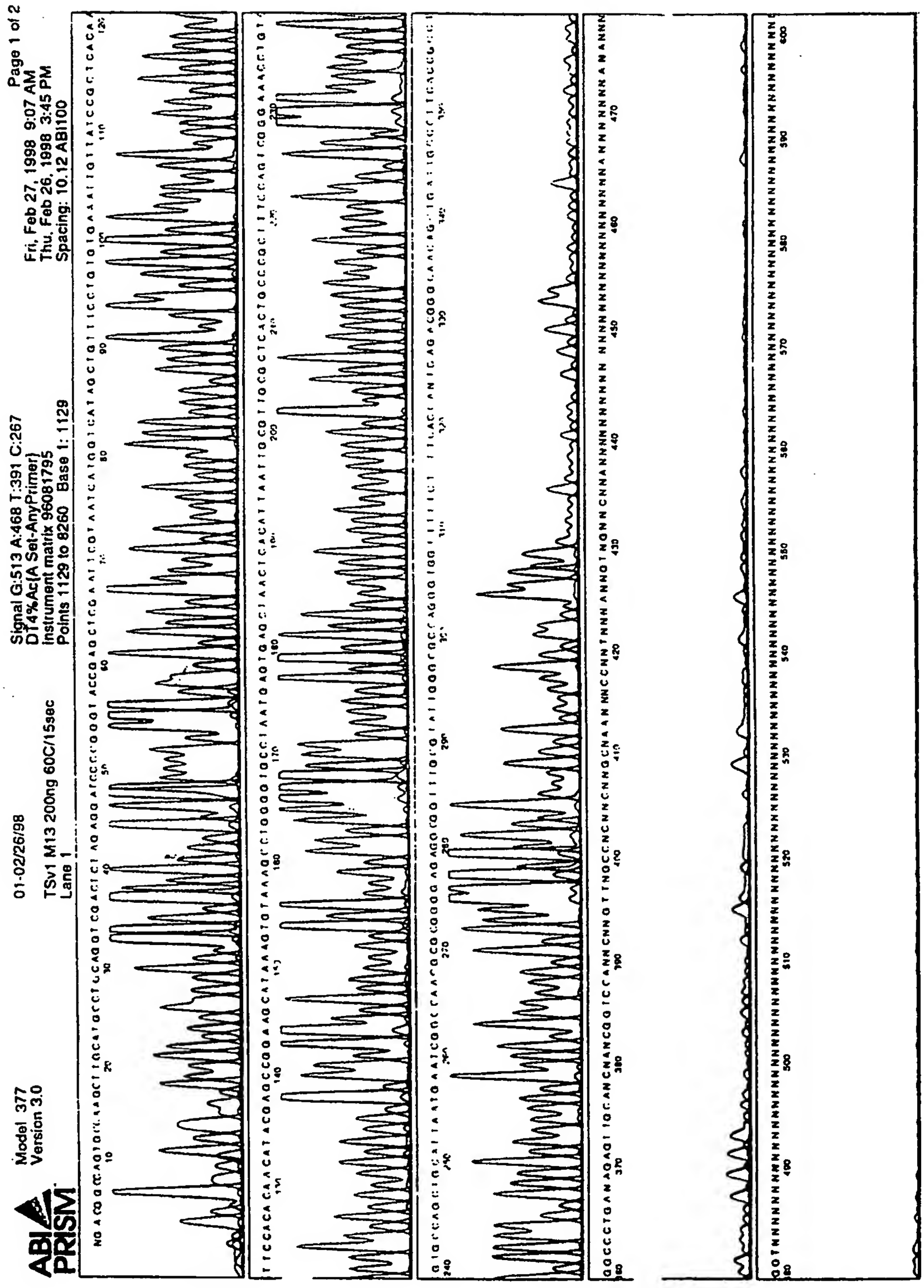
Page 1 of 2
Fri, Feb 27, 1998 9:07 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.12 ABI100
01-02/26/98
TSv1 M13 200ng 60C/15sec
Lane 1
Signal G:513 A:468 T:391 C:267
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1129 to 8260 Base 1: 1129
ABI PRISM
Model 377
Version 3.0

Figure 1B

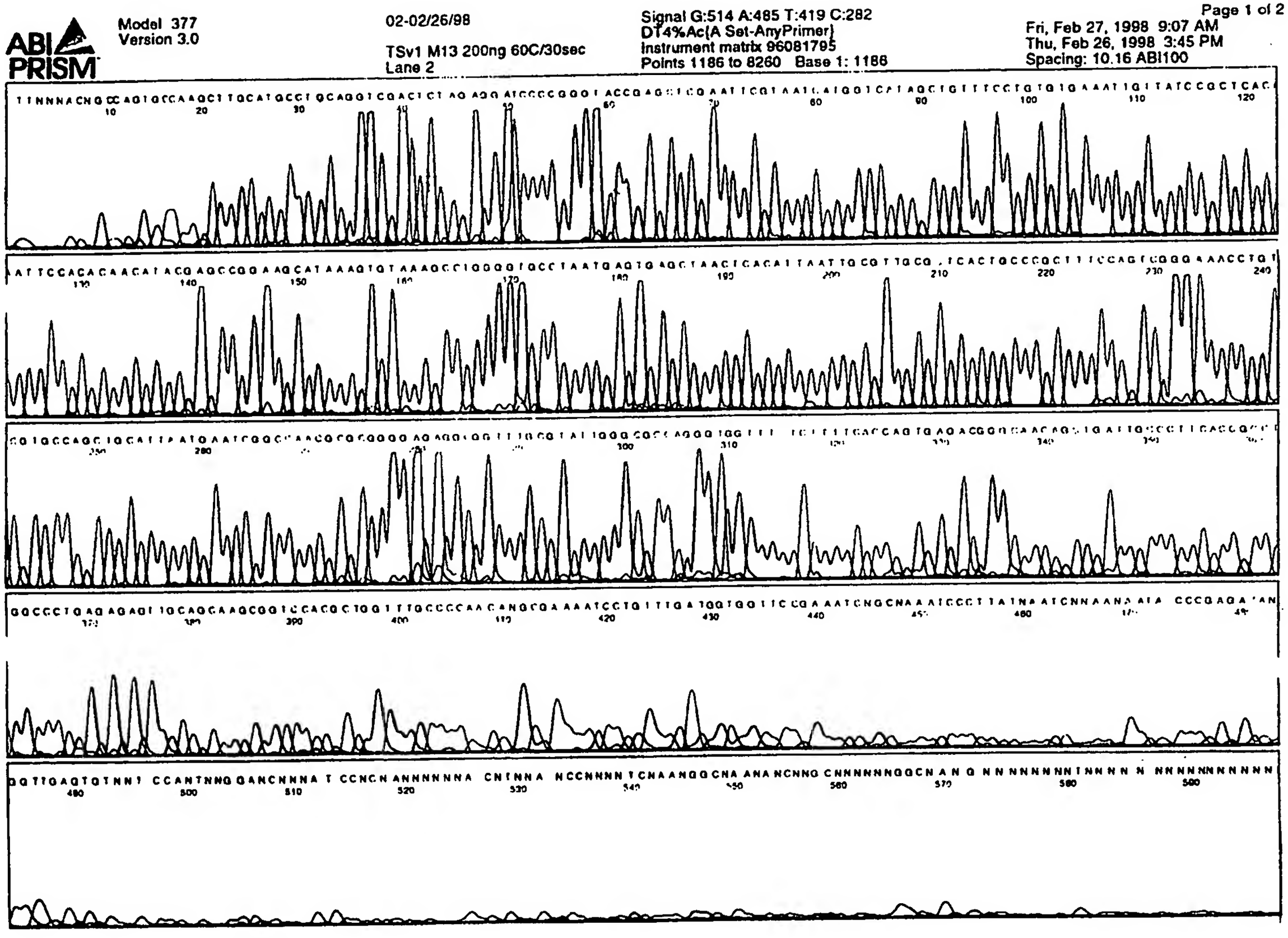
ABI PRISM
Model 377
Version 3.0
02-02/26/98
TSv1 M13 200ng 60C/30sec
Lane 2
Signal G:514 A:485 T:419 C:282
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1186 to 8260 Base 1: 1186
Page 1 of 2
Fri, Feb 27, 1998 9:07 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.16 ABI100

Figure 19

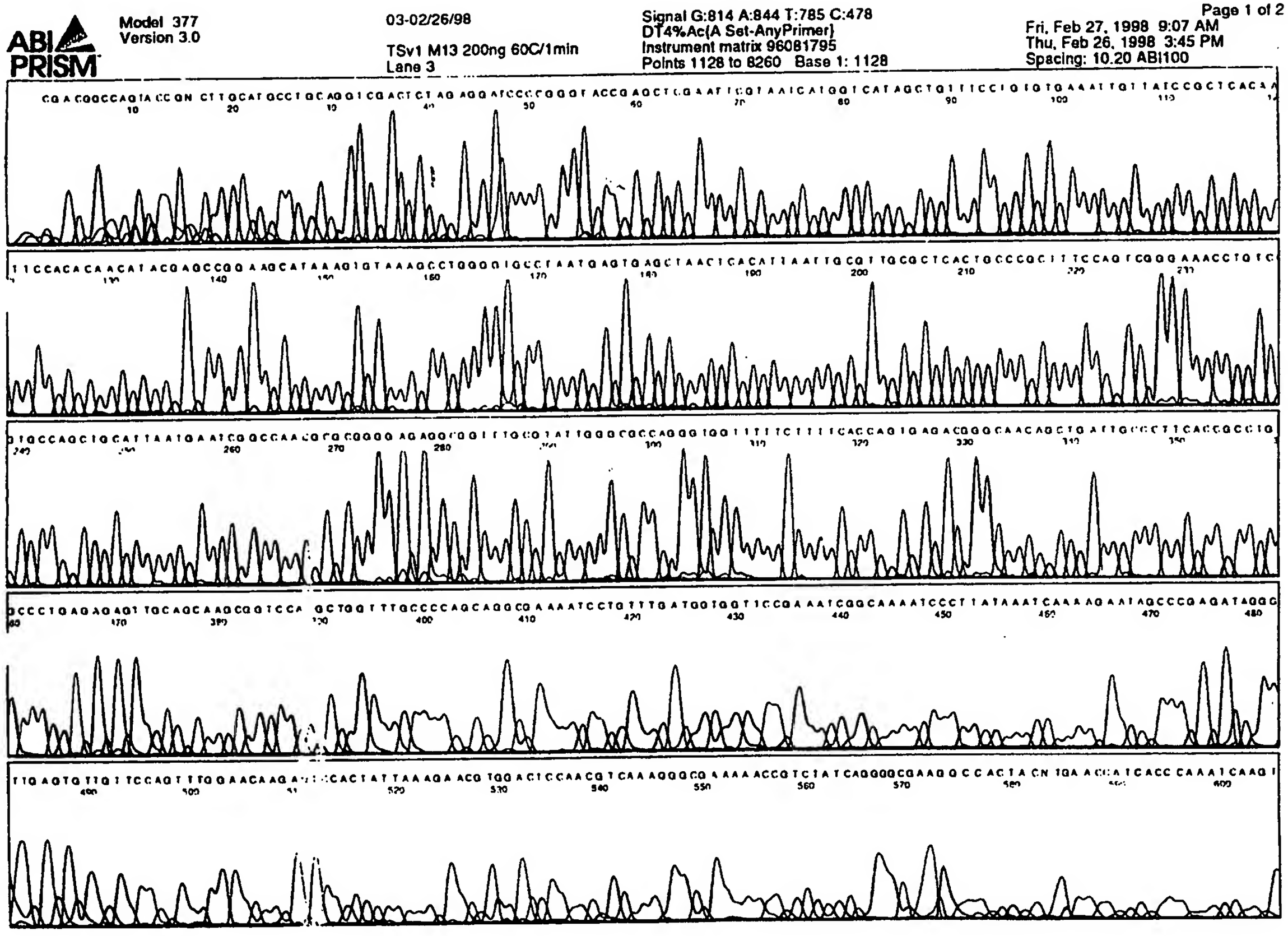
ABI PRISM
Model 377
Version 3.0
03-02/26/98
TSv1 M13 200ng 60C/1min
Lane 3
Signal G:814 A:844 T:785 C:478
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1128 to 8260 Base 1: 1128
Page 1 of 2
Fri, Feb 27, 1998 9:07 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.20 ABI100

Figure 20

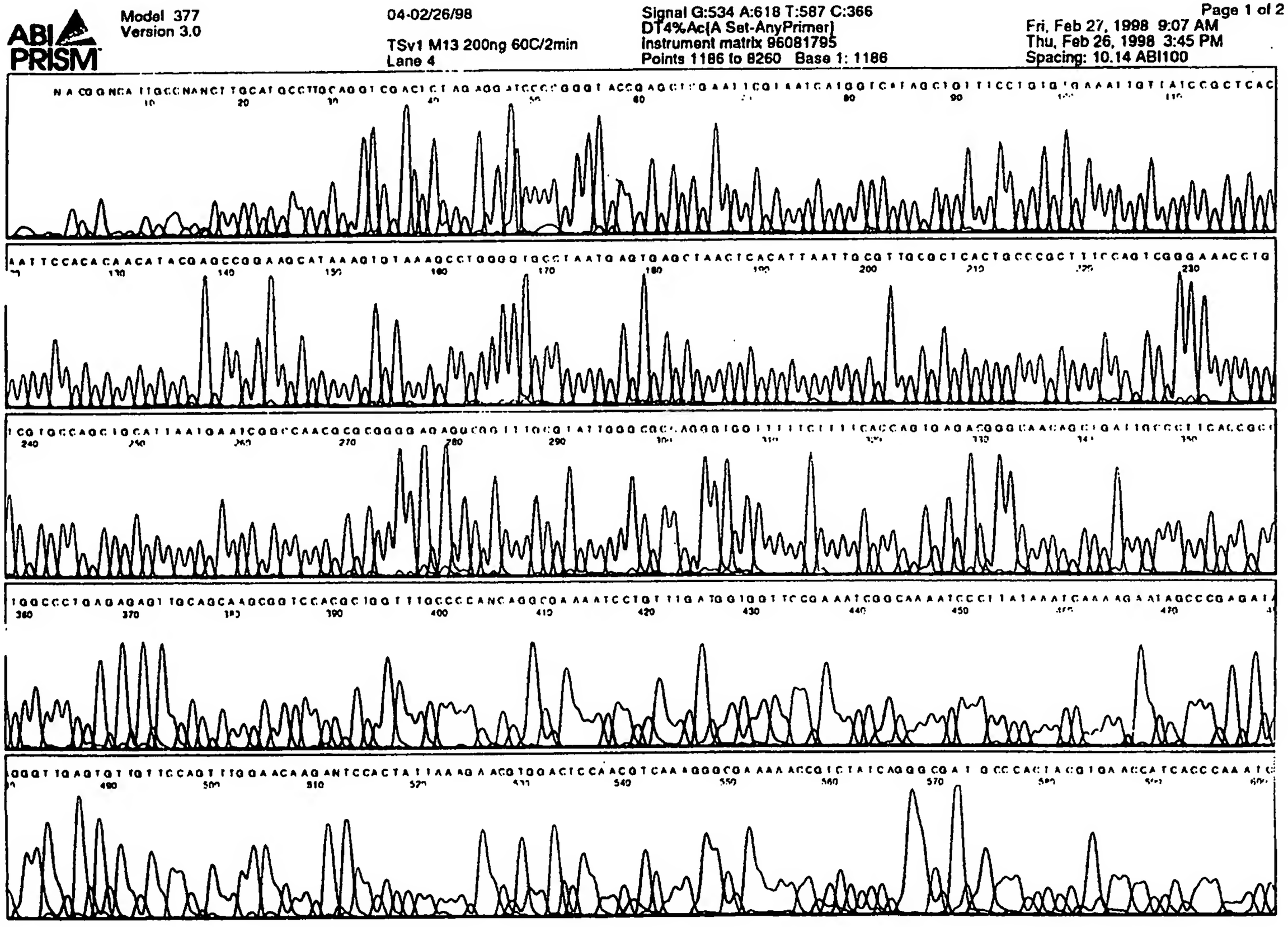
ABI PRISM
Model 377
Version 3.0
04-02/26/98
TSv1 M13 200ng 60C/2min
Lane 4
Signal G:534 A:618 T:587 C:366
DT4%Ac[A Set-AnyPrimer]
Instrument matrix 96081795
Points 1186 to 8260 Base 1: 1186
Page 1 of 2
Fri, Feb 27, 1998 9:07 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.14 ABI100

Figure 21

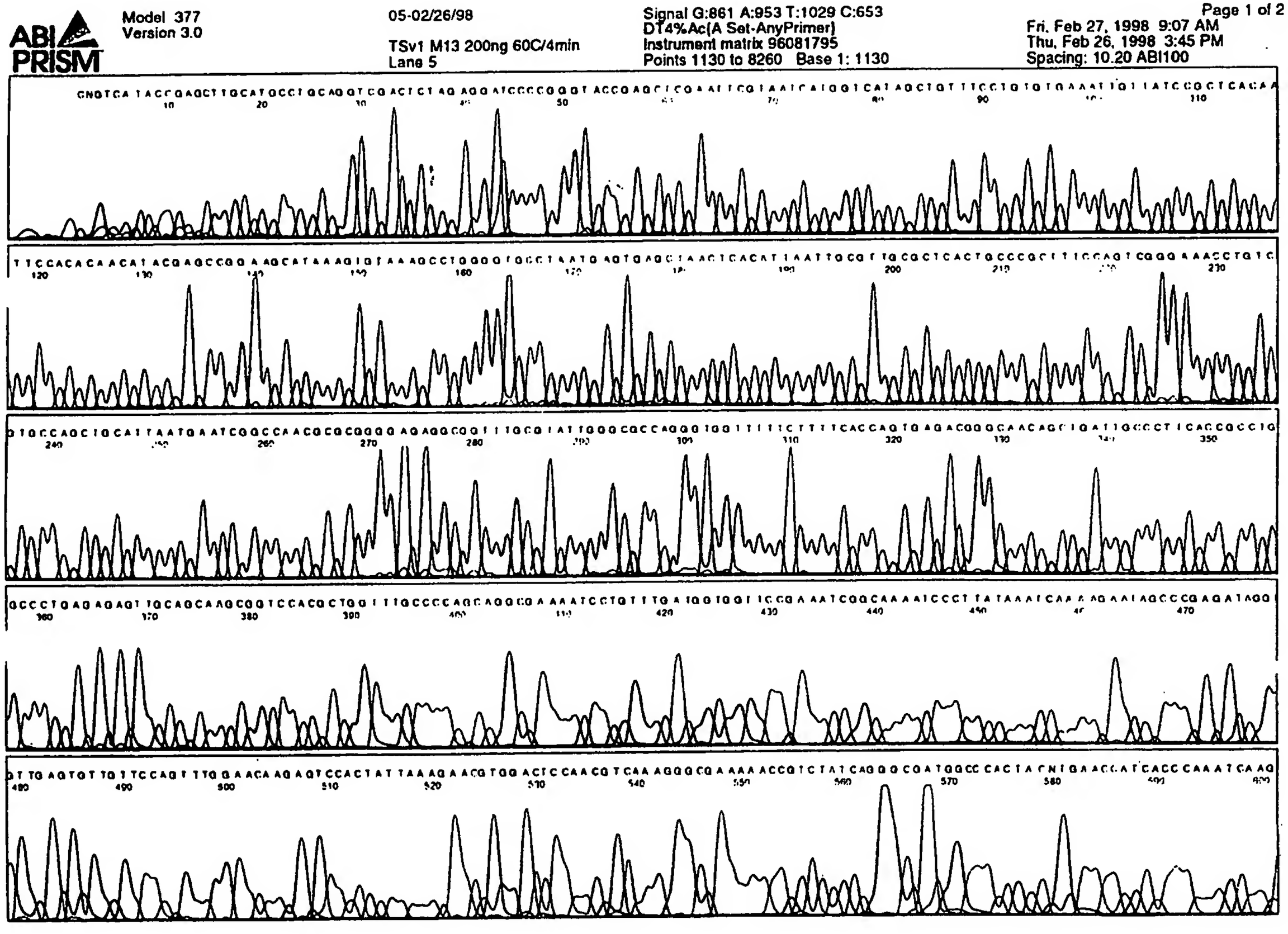
ABI PRISM
Model 377
Version 3.0
05-02/26/98
TSv1 M13 200ng 60C/4min
Lane 5
Signal G:861 A:953 T:1029 C:653
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1130 to 8260 Base 1: 1130
Page 1 of 2
Fri, Feb 27, 1998 9:07 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.20 ABI100

Figure 22

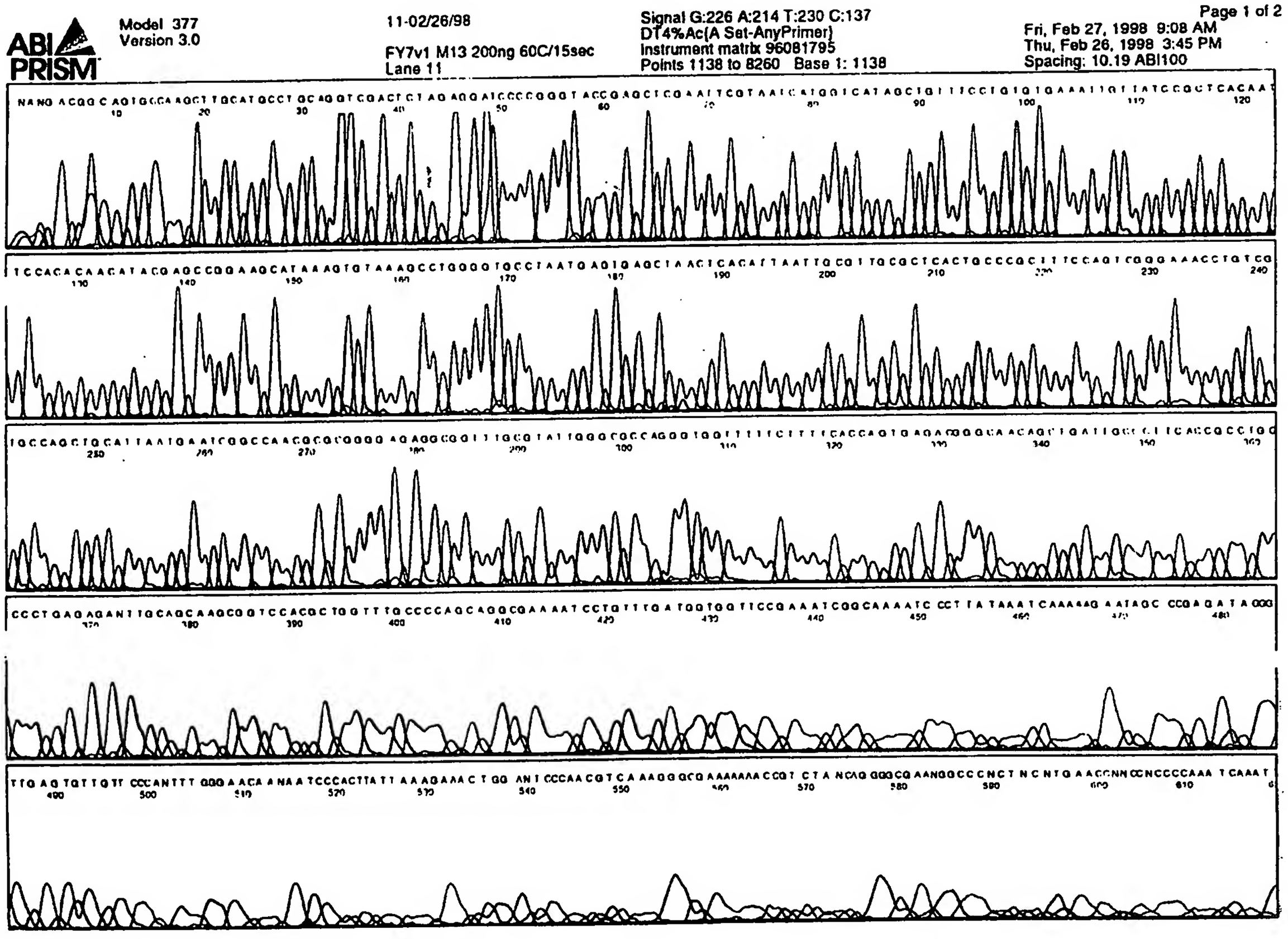
ABI PRISM
Model 377
Version 3.0
11-02/26/98
FY7v1 M13 200ng 60C/15sec
Lane 11
Signal G:226 A:214 T:230 C:137
DT4%Ac{A Set-AnyPrimer}
Instrument matrix 96081795
Points 1138 to 8260 Base 1: 1138
Page 1 of 2
Fri, Feb 27, 1998 9:08 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.19 ABI100

Figure 23

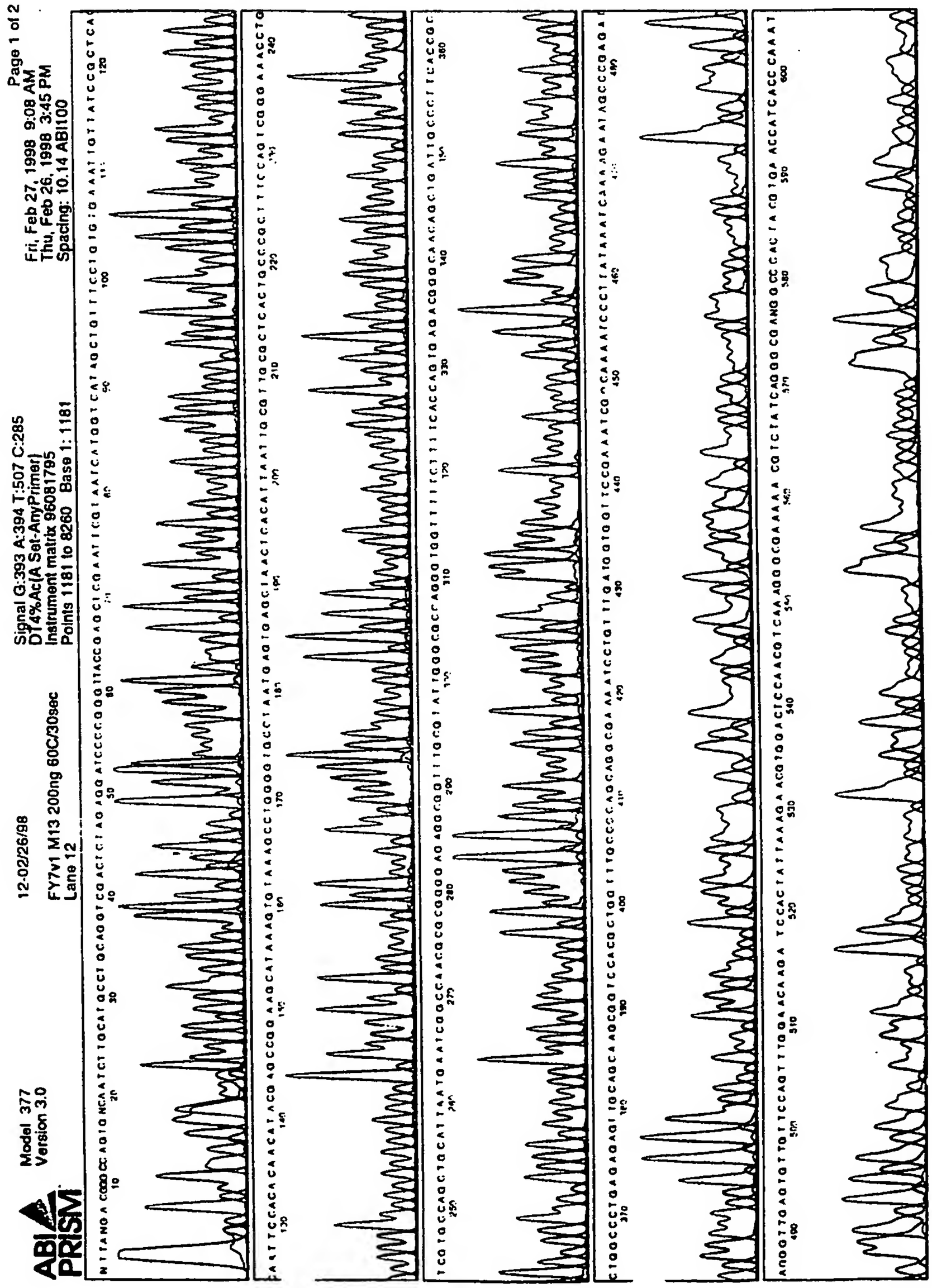
Page 1 of 2
Fri, Feb 27, 1998 9:08 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.14 ABI100
12-02/26/98
FY7v1 M13 200ng 60C/30sec
Lane 12
Signal G:393 A:394 T:507 C:285
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1181 to 8260 Base 1: 1181
ABI PRISM
Model 377
Version 3.0

Figure 24

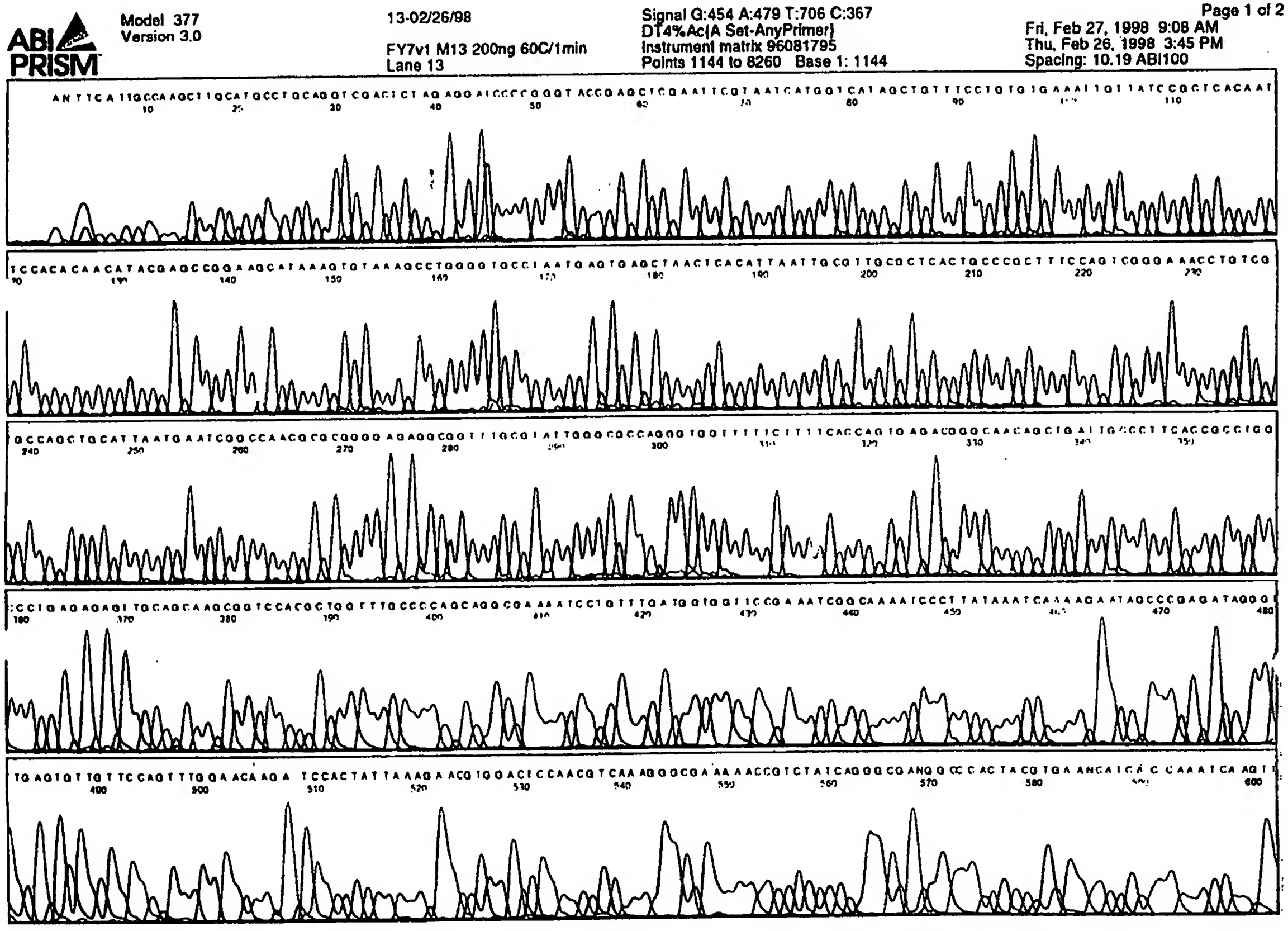
ABI PRISM
Model 377
Version 3.0
13-02/26/98
FY7v1 M13 200ng 60C/1min
Lane 13
Signal G:454 A:479 T:706 C:367
DT4%Ac{A Set-AnyPrimer}
Instrument matrix 96081795
Points 1144 to 8260 Base 1: 1144
Page 1 of 2
Fri, Feb 27, 1998 9:08 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.19 ABI100

Figure 25

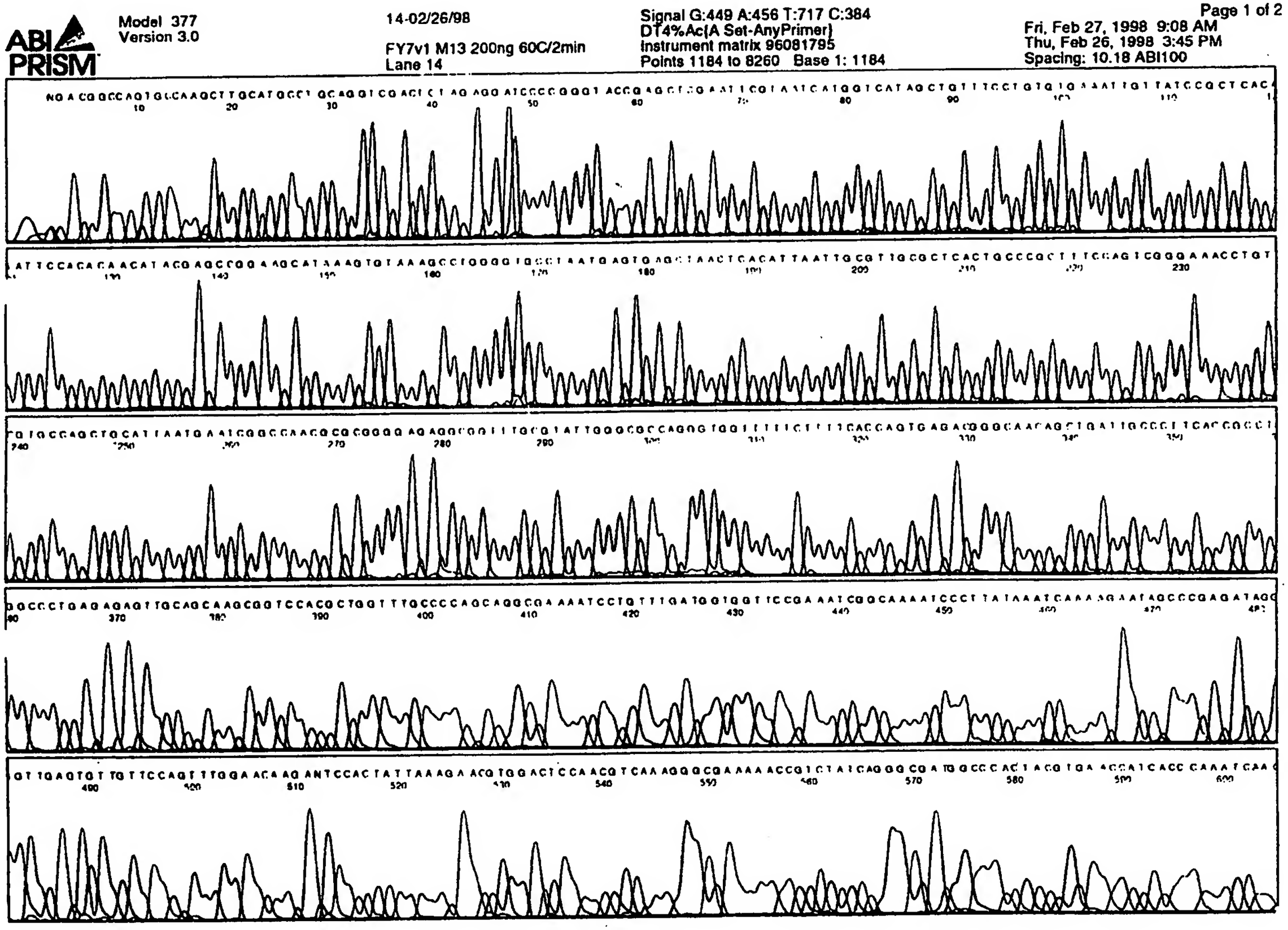
ABI PRISM
Model 377
Version 3.0
14-02/26/98
FY7v1 M13 200ng 60C/2min
Lane 14
Signal G:449 A:456 T:717 C:384
DT4%Ac(A Set-AnyPrimer)
Instrument matrix 96081795
Points 1184 to 8260 Base 1: 1184
Page 1 of 2
Fri, Feb 27, 1998 9:08 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.18 ABI100

Figure 26

ABI PRISM

Model 377
Version 3.0

15-02/26/98

FY7v1 M13 200ng 60C/4min
Lane 15

Signal G:500 A:497 T:843 C:441
DT4%Ac{A Set-AnyPrimer}
Instrument matrix 96081795
Points 1137 to 8260 Base 1: 1137

Fri, Feb 27, 1998 9:08 AM
Thu, Feb 26, 1998 3:45 PM
Spacing: 10.18 ABI100

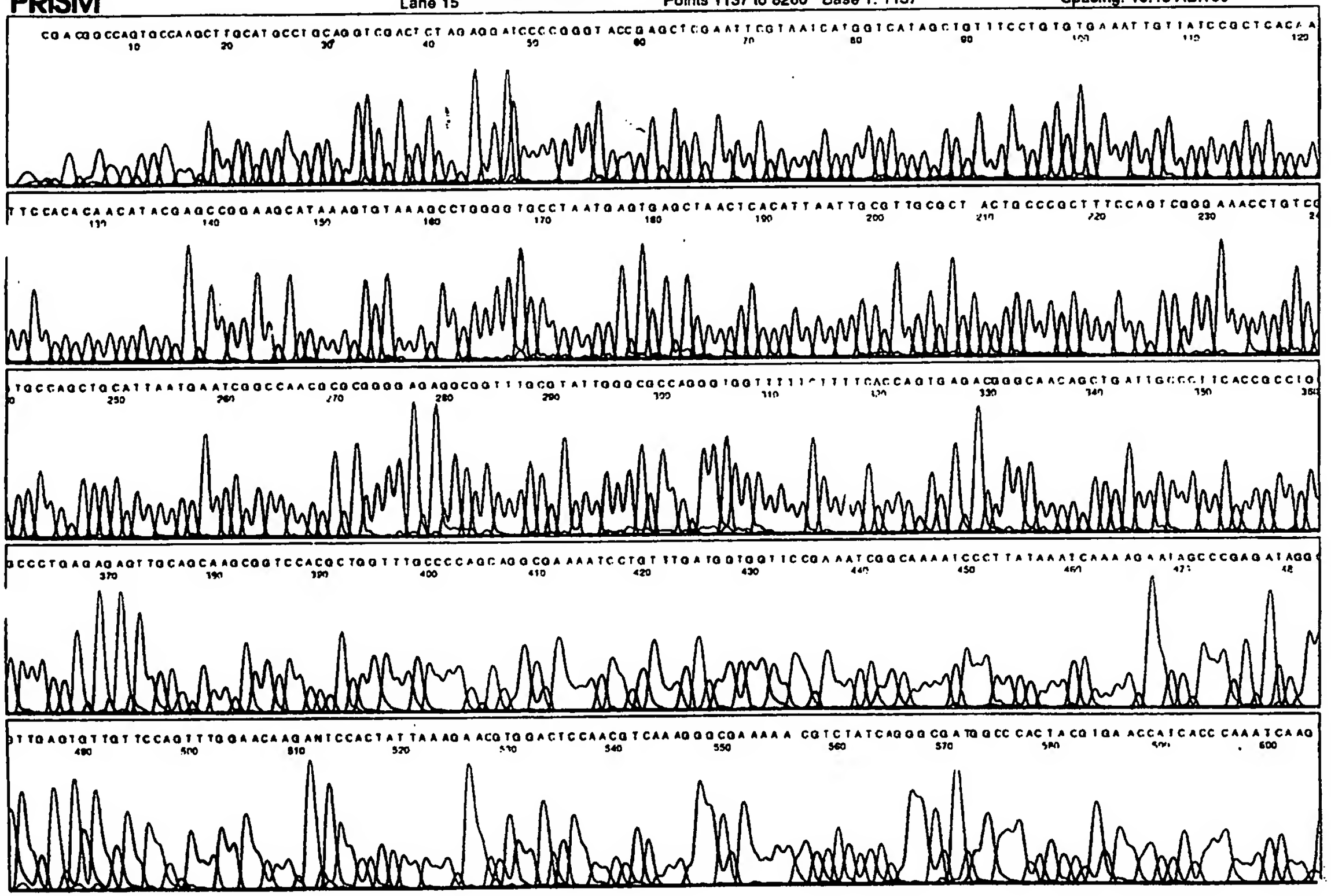

Figure 27

WO 99/65938

PCT/US99/13741